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THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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AUGUST TO DECEMBER, 1939

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THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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C. E. McClung, to serve until 1939.

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MARINE BIOLOGICAL LABORATORY
LIBRARY
WOODS HOLE

II. ACT OF INCORPORATION

No. 3170

Commonwealth of Massachusetts

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, Henry B. Pierce, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W.

G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 11.30 A.M., daylight saving time, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years. There shall be thirty-two Trustees thus chosen divided into four classes, each to serve four years, and in addition there shall be two groups of Trustees as follows: (*a*) Trustees *ex officio,* who shall be the President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer and the Clerk; (*b*) Trustees Emeritus, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next annual meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee Emeritus for life. The Trustees *ex officio* and Emeritus shall have all rights of the Trustees except that Trustees Emeritus shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

II. Special meetings of the members may be called by the Trustees to be held in Boston or in Woods Hole at such time and place as may be designated.

III. Inasmuch as the time and place of the Annual Meeting of Members is fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of said meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President of the Corporation who shall also be Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The accounts of the Treasurer shall be audited annually by a certified public accountant.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

IX. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. THE REPORT OF THE TREASURER

To the Trustees of the Marine Biological Laboratory:

Gentlemen: Herewith is my report as Treasurer of the Marine Biological Laboratory for the year 1938.

The accounts have been audited by Messrs. Seamans, Stetson and Tuttle, certified public accountants. A copy of their report is on file at the Laboratory and is open to inspection by members of the Corporation.

At the end of the year 1938, the book value of the Endowment Funds in the hands of the Central Hanover Bank and Trust Company as Trustee, was

General Fund,	Securities (market $862,409.23)	$ 916,855.70
	Real Estate	9,235.71
	Cash, principal	858.45
Library Fund,	Securities (market $162,008.68)	173,918.24
	Real Estate	20,102.88
	Cash	319.46
		$1,121,290.44

MARINE BIOLOGICAL LABORATORY LIBRARY WOODS HOLE, MASS.

The income collected from these Funds was as follows:

General Endowment	$36,382.94
Library	6,665.66
	$43,048.60

The income in arrears on these Funds at the end of the year was:

Arrears *General Fund*	$13,518.86
Arrears *Library Fund*	3,450.00
	$16,968.86
Arrears at the end of the year 1937	$12,755.86
showing an increase of	$ 4,213.00

The dividends from the General Biological Supply House totalled $14,224.00.

Retirement Fund: A total of $4,060 was paid in pensions of which $197.20 was advanced from current funds. The Fund at the end of the year consisted of mortgages and real estate at the book value of $17,462.08.

Plant Assets: The land (exclusive of Gansett and Devil's Lane), buildings, equipment and library represent an investment of

	$1,789,884.74	
less reserve for depreciation	517,178.00	
or a net of		$1,272,706.74

The hurricane water damage to the inventory and equipment and plant amounted to .... $30,399.02 of which $2,387.97 was charged to Plant Fund and $28,011.05 to Current Surplus. Early this year The Carnegie Corporation of New York most generously contributed $20,000 toward the repair of the hurricane damage.

Income and Expenses: Income including a donation of stock valued at $7,250 exceeded expense, including $24,481.56 depreciation, by $11,432.64.

There was expended from current funds for plant account a net of $15,083.21 and in addition $6,500 in reduction of mortgage and note indebtedness.

At the end of the year the Laboratory owed $5,500 on mortgages and $7,000 on notes all for property purchased in earlier years. It had accounts and notes receivable of $12,305.69 and $8,531.29 in cash and bank accounts in its current funds.

A gift of 200 shares of Crane Company stock was received from Dr. Frank R. Lillie, to which he has since added 300 shares.

Following is the balance sheet, the condensed statement of income and outgo, and the surplus account all as set out by the accountants:

EXHIBIT A

MARINE BIOLOGICAL LABORATORY BALANCE SHEET,

DECEMBER 31, 1938

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of Central Hanover Bank and Trust Company, New York, Trustee—Schedules I-a and I-b		$1,121,290.44	
Securities and Cash—Minor Funds—Schedule II		8,742.81	$1,130,033.25
Plant Assets:			
Land—Schedule IV	$ 110,884.58		
Buildings—Schedule IV	1,239,161.81		
Equipment—Schedule IV	165,567.34		
Library—Schedule IV	274,271.01	$1,789,884.74	
Less Reserve for Depreciation		517,178.00	
		$1,272,706.74	
Cash in Dormitory Building Fund		223.24	
Cash in Reserve Fund		24.65	$1,272,954.63
Current Assets:			
Cash		$ 8,531.29	
Accounts and Notes—Receivable		12,305.69	
Inventories:			
Supply Department	$ 37,672.27		
Biological Bulletin	9,762.64	47,434.91	
Investments:			
Devil's Lane Property	$ 44,398.34		
Gansett Property	5,822.49		
Stock in General Biological Supply House, Inc.	12,700.00		
Other Investment Stocks	7,250.00		
Securities and Real Estate—Retirement Fund List—Schedule V, viz.,			
Retirement Fund Portion	17,264.88		
Current Account Portion	197.20	87,632.91	
Prepaid Insurance		3,193.90	
Items in Suspense (Net)		693.98	$ 159,792.68
			$2,562,780.56

Liabilities

Endowment Funds:			
Endowment Funds—Schedule III	$1,120,581.61		
Reserve for Amortization of Bond Premiums	708.83	$1,121,290.44	
Minor Funds—Schedule III		8,742.81	$1,130,033.25
Plant Liabilities and Funds:			
Mortgage—Payable, Howes Property		$ 5,500.00	
Notes—Payable a/c Bar Neck Property Purchase		7,000.00	
Donations and Gifts—Schedule III		1,038,402.61	
Other Investments in Plant from Gifts and Current Funds		222,052.02	$1,272,954.63
Current Liabilities and Surplus:			
Accounts—Payable		4,077.37	
Reserve for Additional Repairs and Replacements on account of Hurricane Water—Damage		17,518.12	
Current Surplus—Exhibit C.		138,197.19	159,792.68
			$2,562,780.56

EXHIBIT B

Marine Biological Laboratory Income and Expense,

Year Ended December 31, 1938

	Total		Net	
	Expense	Income	Expense	Income
Income:				
General Endowment Fund		$ 36,382.94		$ 36,382.94
Library Fund		6,665.66		6,665.66
Donations		7,250.00		7,250.00
Instruction	8,356.14	9,960.00		1,603.86
Research	4,215.07	16,312.50		12,097.43
Evening Lectures	58.56		58.56	
Biological Bulletin and Membership Dues	9,691.33	10,362.75		671.42
Supply Department—Schedule VI	40,814.01	38,134.93	2,679.08	
Mess—Schedule VII	25,899.67	25,759.83	139.84	
Dormitories—Schedule VIII	22,609.70	12,973.34	9,636.36	
(Interest and Depreciation charged to above 3 Departments —See Schedules VI, VII, and VIII)	*23,731.15*			23,731.15
Dividends, General Biological Supply House, Inc.		14,224.00		14,224.00
Rents:				
Bar Neck Property		3,568.46		3,568.46
Bay Shore Property	206.57	91.75	114.82	
Howes Property	196.64	480.00		283.36
Janitor House	23.19	360.00		336.81
Newman Cottage	81.43	250.00		168.57
Danchakoff Cottage	324.30	750.00		425.70

Sale of Library Duplicates		390.73		390.73
Apparatus Rental		991.30		991.30
Interest on Notes—Receivable		150.00		150.00
Sundry Income		38.54		38.54
Maintenance of Plant:				
Buildings and Grounds	22,482.48		22,482.48	
Chemical and Special Apparatus Expense	14,121.00		14,121.00	
Library Expense	7,576.77		7,576.77	
Truck Expense	1,249.70		1,249.70	
Workmen's Compensation Insurance	507.51		507.51	
Sundry Expense	19.50		19.50	
General Expenses:				
Administration Expense	12,199.94		12,199.94	
Endowment Fund Trustee and Safe-keeping	1,001.95		1,001.95	
Interest on Notes and Mortgage —Payable	829.83		829.83	
Bad Debts	448.39		448.39	
Reserve for Depreciation	24,481.56		24,481.56	
	$173,664.09	$185,096.73	$ 97,547.29	$108,979.93
Excess of income over Expense carried to Current Surplus—Exhibit C	11,432.64		11,432.64	
	$185,096.73		$108,979.93	

EXHIBIT C

Marine Biological Laboratory, Current Surplus Account,

Year Ended December 31, 1938

Balance, January 1, 1938			$153,266.82
Add:			
Excess of Income over Expense for Year as shown in Exhibit B		$11,432.64	
Reserve for Depreciation Charged to Plant Funds		24,481.56	35,914.20
			$189,181.02
Deduct:			
Payments from Current Funds during Year for Plant Assets as shown in Schedule IV,			
Buildings		$ 939.80	
Equipment		4,818.61	
Library		9,499.80	
		$15,258.21	
Less Received for Plant Assets Disposed of		175.00	
		$15,083.21	
Payment on Plant Mortgage and Note—Payable		$ 4,500.00	
Pensions Paid	$4,060.00		

Expenses on Account of Retirement Fund Securities	36.79		
	$4,096.79		
Less Retirement Fund Income and Gain from Security Sale	707.22	3,389.57	
Hurricane Water Damage (except portion Charged to Plant Funds)		28,011.05	50,983.83
Balance, December 31, 1938—Exhibit A			$138,197.19

Respectfully submitted,
LAWRASON RIGGS, JR.,
Treasurer.

V. THE REPORT OF THE LIBRARIAN

A report of the expenditures from the $18,800, appropriated to the Library in 1938, follows: books, $351.67; current serials, $5,319.86; binding, $1,171.38 ($45.00 of this on insurance); express, $181.48; supplies, $1,070.07 (includes $37.53 for new boxes to ship books to the bindery; $707.73 for new catalogue cases); salaries, $7,150.00; back sets, $1,795.33; total, $17,039.79.

For various reasons such as lack of space in the Library and the difficulty of securing the present lacks except in Germany, where prices are high, it seemed best to allow the $1,760.21 available for back sets besides $390.73 for the Library sale of duplicates, to revert to the General Fund of the Laboratory. Also a correction of the printed 1937 report must be made here. An order for the back set of "Flora" placed in Germany failed to come through and the order was finally cancelled by the Librarian, allowing another sum of $2,430.50 to drop from the Library expenditures.

The usual appropriation to the Library of $600.00 by the Woods Hole Oceanographic Institution was expended to the amount of $591.98 and separately accounted.

This year the Library lists but 1,306 current serials of which 426 are subscriptions, 385 (11 new) purchases of the Marine Biological Laboratory, 41 (1 new) of the Woods Hole Oceanographic Institution; 666 are exchanges, 596 (4 new) with the Biological Bulletin and 70 (1 new) with the Woods Hole Oceanographic Institution publications; and 207 come as gifts to the former and 7 as gifts to the latter. The record shows 47 books purchased, 41 by the Marine Biological Laboratory and 6 by the Woods Hole Oceanographic Institution, 19 presented by the authors and 41 from publishers; while a contribution from Dr. Alfred Meyer enabled the Library to purchase a new "American Medical Directory"; and Dr. Douglas M. Whitaker presented a copy of Beaumont's "Experiments and Observations on the Gastric Juice and the Physiology of Digestion." Completed back sets of serials number 36; as purchases of the Marine Biological Laboratory, 20, of the Woods Hole Oceanographic Institution, 2; while purchases partially completing back sets number 15 for the former and 1 for the latter; through exchange of duplicates, 11 completed back sets for the former, and 1 for the latter; besides many additions to still incomplete back sets; and 2 sets for the Marine Biological Laboratory completed by gifts, with 4 partially completed. Reprint additions number 6,905: current for 1937, 1,897; current for 1938, 894, and of date previous to 1937, 4,114; about 200 of the latter kindly presented by Dr. M. A. Bigelow and 70 by Mrs. H. H. Donaldson. A summary of the current holdings of the Library proper is therefore 44,897 bound volumes and 108,927 reprints.

VI. THE REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen: I beg to present herewith a report of the fifty-first session of the Marine Biological Laboratory for the year 1938.

1. *Attendance.* The number of investigators and their assistants present during the summer of 1938 was somewhat less than in 1937, but it taxed the facilities of the Laboratory to the utmost. Attendance has risen steadily, with minor fluctuations, since 1933; now the number present is greater than the optimum which can be cared for under existing conditions. We are rapidly approaching the time when selection among the applicants for research space must be made, a situation referred to in the report of the Committee on Future Policy in the following words: "It will be necessary to adopt more definite policies concerning the admission of investigators than in the past. These should not, however, be of too binding a character, but rather a definition of principles within which the Director will have free scope for the exercise of his best judgment." The definition of these principles deserves the most careful consideration.

2. *The Library.* The continued growth of the Library is a source of satisfaction to the investigator, but it presents a serious problem to the Librarian who must find a place for new volumes and reprints. Each year's increment of bound volumes requires a space about equal to one complete stack. Since the present stacks are already practically filled, it will presently be necessary to use rooms now employed for cataloguing or other Library purposes. This will disturb the present orderly arrangement of serials and will at best provide only temporary relief. An addition to the Library is urgently needed.

3. *The Board of Trustees.* At the meeting of the Corporation held Tuesday, August 9, 1938, Dr. H. S. Jennings, Trustee since 1905, was elected Trustee Emeritus. To fill his place in the Class of 1942, Dr. M. H. Jacobs, the retiring Director, was chosen. At the same meeting, Dr. P. H. Armstrong was elected Clerk of the Corporation in place of Dr. Charles Packard who resigned when appointed Assistant Director.

The Board has suffered heavy losses by death. Mr. Charles R. Crane, Trustee from 1901 and President of the Board from 1902 to 1925, " the best friend the Laboratory ever had "; Dr. Edmund B. Wilson, Trustee continuously from 1890, whose contributions from this Laboratory were instrumental in establishing its scientific eminence; Dr. Charles R. Stockard, Trustee from 1920, whose counsels, vigorously expressed, were always highly valued; and Dr. J. Playfair McMurrich, Trustee from 1892 to 1900, active in the early days of this Laboratory.

4. *The Hurricane and Flood.* We may be profoundly thankful that in the storm of September 21, 1938 no one connected with the Laboratory lost his life. Some were rescued from desperate situations, and many suffered heavy material loss. Damage to Laboratory property was due almost entirely to water which poured into the basement of the Brick Building, into the Supply Department and the Dormitory, washed away most of the foundations of the Club House, and carried the Bathhouse far inland. The old laboratory buildings and the Mess were above the flood level.

During the height of the storm our staff worked heroically to protect the buildings and equipment. Mr. Larkin organized a bucket brigade and saved the apparatus in the Pump House; Mr. McInnis and his crew protected the motor boats; others barricaded doors in the Brick Building against the rising waters, but to no avail for the flood broke through the windows in the sub-basement of the Library; Mr. MacNaught opened the Apartment House to those who had been driven from their homes.

The greatest loss occurred in the Brick Building where the water, four feet deep, submerged microscopes and electrical apparatus, overran the storage battery, the switchboard and motors, and reduced the chemical and storage rooms to utter confusion.

The work of repair began at once. Dr. Pond and his assistants examined all the apparatus which had been wet with salt water, reconditioned much of it in our workship, and sent some to the manufacturers for servicing. To restore the Chemical Room required many weeks of hard work. Mr. McInnis and his men quickly reduced the confusion in the Supply Department where the damage was not great, and were able, within a few days, to resume regular business. Under the direction of Mr. Larkin, the storage battery was cleaned and recharged, and the various motors were dried and set in place. None were lost, but some needed repairs. The switchboard was damaged but has been reconditioned. Mrs. Montgomery saved many of the more important duplicate reprints which had been water-soaked. Fortunately the regular reprint collection and the bound volumes were never in danger. The bathhouse, after being put back on new foundations, was damaged by a second storm. By order of the Executive Committee it was removed entirely.

These very extensive repairs to the buildings and the equipment have been made almost entirely by our permanent staff who have given unsparingly of their time and energy. To them the Laboratory owes a debt of gratitude.

In the Treasurer's Report the loss due to the storm is set at $30,400. This sum includes all of the various items which were lost. Inasmuch as many of these were of little actual value, and need not be replaced, the actual cost of restoring the damage will undoubtedly be less than $25,000. Since the Laboratory carried no insurance against this type of loss, the financial burden thus imposed upon us was serious. But we are fortunate in our friends. The Carnegie Corporation of New York, a benefactor of former years, has presented to the Laboratory the sum of $20,000 to be used for purposes of restoration. We are sincerely grateful for this generous and timely gift.

5. *Research in Botany.* For some years it has been apparent that the number of investigators at the Laboratory carrying on research in Botany has declined. This situation is due in part to the fact that some of the members of the Research Staff have been unable to attend the summer session, and in part to the lack of facilities for pursuing research in the dynamic phases of Botany. Following the resignation of Drs. Ivy M. Lewis, C. E. Allen and W. J. Robbins from the staff after many years of active service, Dr. E. W. Sinnott, of Columbia University, and Dr. D. R. Goddard, of the University of Rochester, were appointed. The lack of facilities for research has been stressed by many botanists who have expressed the opinion that more laboratory space is needed, that a suitable plot of ground for raising plants should be provided, and that a greenhouse is an essential part of an active botanical laboratory. These requirements should be met at the earliest opportunity.

6. *Gifts.* The sum of $20,000 given by the Carnegie Corporation of New York, to be used for the purpose of restoring the damage done by the flood, has already been mentioned. The Marine Biological Laboratory also gratefully acknowledges gifts amounting to $17,775 presented by Dr. F. R. Lillie.

7. *The Committee on Future Policy.* At the meeting of August 11, 1937, the Board of Trustees authorized the President to appoint a committee to formulate a statement concerning the policies and future of the Marine Biological Laboratory. The members of this Committee are: E. G. Conklin, Chairman, G. N. Calkins, W. C. Curtis, H. B. Goodrich, M. H. Jacobs, T. H. Morgan, G. H. Parker, A. C. Redfield and C. R. Stockard. After many discussions during the summers of 1937 and 1938 a report was drawn up by Dr. Lillie. This was studied and amended by the Committee and is now presented on p. 15 of this Annual Report.

8. *Lectures and Scientific Meetings.* During the summer of 1938 there were ten regular evening lectures and seven seminars at which shorter papers were discussed. In addition to these there were several informal exhibitions of motion pictures of scientific interest and a number of discussion groups. At the final scientific meetings, held August 30 and August 31, numerous investigators reported the results of their work during the current summer. In addition, many demonstrations were on display, both at the Laboratory and at the Fish Commission.

One of the regular seminar evenings was devoted to an informal celebration of the fiftieth anniversary of the founding of the Laboratory. Dr. Conklin reviewed the history of the early days, and Dr. Lillie spoke of those who have contributed to the scientific and material welfare of the institution. At the close of the meeting he presented to the Laboratory, in behalf of the Trustees, a portrait of Mr. Crane. It was a great source of satisfaction that Mr. Crane could be present to receive greetings from his many friends.

As in previous years, the Laboratory was host to the Genetics Society of America, which held its meetings on August 31 and September 1.

There are appended as parts of the report:

1. The Report of the Committee on Policies and Future of the Marine Biological Laboratory.
2. The Staff, 1938.
3. Investigators and Students, 1938.
4. A Tabular View of Attendance, 1934–38.
5. Subscribing and Coöperating Institutions, 1938.
6. Evening Lectures, 1938.
7. Shorter Scientific Papers, 1938.
8. General Scientific Meeting, 1938.
9. Members of the Corporation, 1938.

Respectfully submitted,

CHARLES PACKARD,

Associate Director.

1. REPORT OF THE COMMITTEE APPOINTED ON REQUEST OF THE BOARD OF TRUSTEES, AUGUST 10, 1937, TO FORMULATE A STATEMENT CONCERNING THE POLICIES AND FUTURE OF THE MARINE BIOLOGICAL LABORATORY

I. Introduction

By way of introduction, it is important to remind ourselves of the aims of the founders of the Marine Biological Laboratory. For this purpose a series of quotations follows. It is not the intention to present a history in any detail because it will be found that the original statements of policies and aims have been carefully observed during the entire history of the Laboratory for the fifty years of its existence. As the first director early remarked, " These policies should be the germ of an indefinite future development "; and this has been the case.

In the First Annual Report of the Marine Biological Laboratory for the year 1888, the Trustees made the following statements:

> " *Foundation.*—The Marine Biological Laboratory is an outgrowth of a sea-side laboratory maintained at Annisquam, Mass., from 1880 to 1886, by the Women's Education Association of Boston, in coöperation with the Boston Society of Natural History. In 1886, efforts were made by the Association to place the Laboratory on an independent and broader foundation. A circular letter was addressed to many of the leading biologists of the country, reciting what had been already done at Annisquam, and asking for coöperation and counsel. The replies received were most encouraging, testifying to a general and hearty approval of the enterprise, and promising coöperation and support." (P. 7.)
>
> " At the first meeting held by this committee, its members showed by votes that it was their desire to found a laboratory that should give opportunity for original research as well as for instruction, and soon after appointed the following
>
> Trustees
>
> Prof. William G. Farlow, Prof. Charles S. Minot,
> Miss Florence M. Cushing, Miss Susan Minns,
> Prof. Alpheus Hyatt, Prof. William T. Sedgwick,
> Mr. Samuel Wells." (P. 8.)

The first announcement issued in 1888 contained the following statements:

> " The Trustees of the Marine Biological Laboratory earnestly desire to enlist your co-operation in the support of a sea-side laboratory for instruction and investigation in Biology."
>
> " It is the desire of the Trustees that the enterprise shall enlist the active support of the universities and colleges of the country. To pre-

> vent its becoming a simply local undertaking, they wish to see all who aid in its support by subscribing to investigators' tables share with the other members of the Corporation in the annual election of Trustees. The Trustees will, therefore, invite each institution which holds an investigator's table to name five persons for members of the Corporation during the term of subscription."

Dr. Whitman commented on these statements in the Eighth Annual Report, for the year 1895 as follows:

> "Here we see sketched the elemental basis of our germ-organization —mainly potentialities of a theoretical nature, but 'instinct with spirit.' The aim was a permanent biological station; the function was to be instruction and investigation; the formative principle relied upon was co-operation." (P. 19.)

Whitman himself was the most influential person in determining the policies and aims of the new laboratory. In his first annual report as Director in 1888 he stated his personal viewpoint as follows:

> "The new Laboratory at Woods Hole is nothing more, and, I trust, nothing less, than a first step towards the establishment of an ideal biological station, organized on a basis broad enough to represent all important features of the several types of laboratories hitherto known in Europe and America. It should be provided eventually with means for sending men to different points of the coast to undertake the investigation of subjects of special interest, thus adding to the advantages of a fixed station those of an itinerant laboratory.
>
> "The research department should furnish just the elements required for the organization of a thoroughly efficient department of instruction. Other things being equal, the investigator is always the best instructor. The highest grade of instruction in any science can only be furnished by one who is thoroughly imbued with the scientific spirit, and who is actually engaged in original work. Hence the propriety—and, I may say, the necessity—of linking the function of instruction with that of investigation. The advantages of so doing are not by any means confined to one side. Teaching is beneficial to the investigator, and the highest powers of acquisition are never reached where the faculty of imparting is neglected. Teaching is an art twice blest; it blesseth him that gives and him that takes. To limit the work of the Laboratory to teaching would be a most serious mistake; and to exclude teaching would shut out the possibilities of the highest development. The combination of the two functions in mutually stimulating relations is a feature of the Laboratory to be strongly commended." (Pp. 16–17.)

In his lecture on "Specialization and Organization" (Biological Lectures, 1890) he remarked:

> "Among the ways of bringing together our scattered forces into something like organic union, the most important, and the most urgent at

> this moment, is that of a national marine biological station. Such an establishment, with a strong endowment, is unquestionably the great desideratum of American biology. There is no other means that would bring together so large a number of the leading naturalists of the country, and at the same time place them in such intimate helpful relations to one another. The larger the number of specialists working together, the more completely is the organized whole represented, and the greater and the more numerous the mutual advantages." (P. 24.)

In 1893 he wrote in his lecture on " Work and Aims of the Marine Biological Laboratory " (Biological Lectures, 1893) :

> " To those who by word and example have encouraged coöperation, this record will certainly be gratifying; and perhaps it will be accepted by all as an assurance that good-will and united effort have not been fruitless. For six years the Marine Biological Laboratory has stood for the first and the only coöperative organization in the interest of Marine Biology in America." (P. 236.)

The same year he remarked in his article " A Marine Observatory the Prime Need of American Biologists " (Atlantic Monthly, June, 1893, pp. 808–815) :

> " The Marine Biological Laboratory attaches itself to no single institution, but holds itself rigidly to the impartial function of serving all on the same terms. It depends not upon one faculty for its staff of instructors, but seeks the best men it can find among the higher institutions of the land. The board of trustees is a growing body, every year adding to its number, until it now comprises a very large proportion of the leading biologists of America. The whole policy is national in spirit and scope. The laboratory exists in the interest of biology at large, and not to nurse the prestige of any university or the pride of individual pretension." (P. 811.)
>
> " Representative character, devotion to biology at large, independent government,—such are the essential elements of a strong and progressive organization." (P. 812.)

Again in 1898 he returned to the theme in an article " Some of the Functions and Features of a Biological Station " (*Science,* N.S., Vol. 7, No. 159, January 14, 1898, pp. 11–12) :

> " It now remains to briefly sketch the general character and to emphasize some of the leading features to be represented in a biological station.
>
> " The first requisite is capacity for growth in all directions consistent with the symmetrical development of biology as a whole. The second requisite is the union of the two functions, research and instruction, in such relations as will best hold the work and the workers in the natural coördination essential to scientific progress and to individual development. It is on this basis that I would construct the ideal and test every practical issue.

> " A scheme that excludes all limitations except such as nature prescribes is just broad enough to take in the science, and that does not strike me as at all extravagant or even as exceeding by a hair's breadth the essentials. Whoever feels it an advantage to be fettered by self-imposed limitations will part company with us here. If any one is troubled with the question: Of what use is an ideal too large to be realized? I will answer at once. It is the merit of this ideal that it can be realized just as every sound ideal can be realized, only by gradual growth. An ideal that could be realized all at once would exclude growth and leave nothing to be done but to work on in grooves. That is precisely the danger we are seeking to avoid.
>
> " The two fundamental requisites which I have just defined scarcely need any amplification. Their implications, however, are far-reaching, and I may, therefore, point out a little more explicitly what is involved. I have made use of the term 'biological station' in preference to those in more common use, for the reason that my ideal rejects every artificial limitation that might check growth or force a one-sided development. I have in mind, then, not a station devoted exclusively to zoology, or exclusively to botany, or exclusively to physiology; not a station limited to the study of marine plants and animals; not a lacustral station dealing only with land and fresh-water faunas and floras; not a station limited to experimental work, but a genuine biological station, embracing all these important divisions, absolutely free of every artificial restriction.
>
> " Now, that is a scheme than can grow just as fast as biology grows, and I am of the opinion that nothing short of it could ever adequately represent a national center of instruction and research in biology. Vast as the scheme is, at least in its possibilities, it is a true germ, all the principal parts of which could be realized in respectable beginnings in a very few years and at no enormous expense. With scarcely anything beyond our hands to work with, we have already succeeded in getting zoology and botany well started at Woods Hole, and physiology is ready to follow."

II. Future Plans and Policies

A. The Problem of Expansion vs. Consolidation

Since the erection of the " New Laboratory " in 1923, there has been a steady growth in the attendance of investigators, subject to some recession during the depression, but reaching a peak in 1937 which strained our accommodations to the limit during the greater part of the session. The question is therefore forced upon our attention whether we should limit arbitrarily the number of investigators as we have long since done in the case of students in classes. The only alternative would be to increase our accommodations. Decision of this point would affect various policies, and it should therefore receive first consideration, The Committee have given careful attention to the question of expansion and have reached the unanimous conclusion that it would be wise at this time to consolidate and develop our present plant and organization, and to postpone the question of expansion, or of new construction except as noted below under Library and under Instruction.

The main reasons for this opinion are two: first, that the problems of housing and adequate care of a considerably larger number of persons would be difficult in the restricted community in which we find ourselves, and second, the need of prudence which rests upon economic uncertainties. It is by no means certain that we may not have to face another period of depression before many years, and this should not find us over-expanded. Each of these considerations can, of course, be developed in detail.

B. The Principle of Coöperation

Whitman spoke of coöperation as the "formative principle" of the Laboratory. It is illustrated in the national scope of the Laboratory and in its fundamental organization and government. The principles involved in nation-wide institutional representation and coöperation, and in comprehensive membership of the Corporation, are so rooted in our practices and have proved so fruitful as to require only emphasis.

C. Organization and Government

The inter-relations of Trustees and Corporation as given in the By-laws have operated harmoniously and effectively for a long time.

Rules concerning nomination and election of Trustees and members of the Corporation by the respective bodies have been formulated as follows:

1. By the Corporation:—August 11, 1931.

> 1) After considering various methods by which those engaged in instruction might be represented upon the Board of Trustees, it is believed that the following action by the Corporation will be the best means of insuring such representation:
>
> "The Corporation affirms its position that instruction in course work is a fundamental part of the work of the Laboratory and should be adequately represented upon the Board of Trustees."
>
> 2) "That the Committee of the Corporation for nomination of Trustees consist of five members, of whom not less than two shall be non-Trustee members and not less than two shall be Trustee members of the Corporation."

3) " That on or about July first of each year, the Clerk shall send a circular letter to each member of the Corporation giving the names of the Nominating Committee and stating that this committee desires suggestions regarding nomination."

4) " That the Nominating Committee shall post the list of nominations at least one week in advance of the annual meeting of the Corporation."

(Memo: The same committee also makes nominations annually for Treasurer and Clerk of the Corporation.)

2. By the Trustees:—August 10, 1937.

" Proposals for membership in the Corporation shall be made to the Nominating Committee on or before the first Tuesday of August upon a regular form and endorsed by two members of the Corporation.

" With the recognition that rigid and completely standardized requirements for membership in the Corporation of the Marine Biological Laboratory are neither practicable nor desirable, it is recommended that future members of the Corporation shall, in general, be selected from among persons who, by engaging in active research at the Marine Biological Laboratory during substantial portions of at least two summers, shall have become acquainted with the work, aims, and peculiar problems of the Laboratory, and who, by papers published over a period of several years shall have demonstrated a capacity for sustained scientific productiveness not less than that required for full membership in such national societies as the American Society of Zoölogists, the Botanical Society of America, and the American Physiological Society.

" It is further recommended that in doubtful or border-line cases action on applications for membership shall be deferred until a time when, in the opinion of the Nominating Committee then serving, the status of the applicant has become entirely clear."

D. Administration

In the course of the years we have developed methods of administration of the various service departments of the Laboratory that have worked well. It should be the function of the Director and Assistant Director to control the operation of such services.

Dr. Jacobs' greatly regretted resignation as Director raises very directly the question of the higher administration. The first two Directors of the Laboratory served without salary, and the routine administration was performed by an Assistant Director on pay, at first part time but later on full time. Then Dr. Jacobs performed the services both of Director and Assistant Director on half time and half pay, and the Business Manager became able with experience to take over many of the duties formerly exercised by the Assistant Director. Though this arrangement worked admirably for the period of its duration, experience showed that it is not reasonable to expect a man of the scientific experience and reputation expected of the Director of this Laboratory to endure indefinitely the limitations of scientific activity imposed by such an arrangement. It seems probable that we cannot return to this plan.

As soon as possible we should provide for a full-time resident Director or Assistant Director. This would afford continuous supervision of the business of the Laboratory and in addition would permit this officer to continue his research work under favorable conditions. Such a resident scientist would attract other scientists during the portion of the year when the Laboratory is little used and would thus help to make it an all-year-round institution.

E. Research and Instruction

Research and instruction have been companion principles since the foundation of the Laboratory as cited in the introduction to this report. In the maintenance of research and instruction side by side throughout its history, the Marine Biological Laboratory has been outstanding, if not strictly unique. We have stood by the principle that it is the business of the Laboratory to help to produce investigators as well as investigation; and we believe that it can be shown that our courses of instruction have contributed in an important way to this purpose, and, moreover, that they have been an important factor in the improvement of biological instruction and research throughout the country. Although there has been some opinion among members of the Laboratory since the courses ceased to be an important source of income that we would be better off without courses, this opinion has never prevailed. We believe that our problem is in the way of improvement, not elimination, of instruction.

The Laboratory has no program of its own in research, except as defined in its name, and it therefore promotes no specific research projects as official undertakings. It operates entirely on the principle of furnishing facilities to competent investigators, and to beginning investigators who are working under qualified direction. No biological subjects are specifically excluded except such as are ruled out by lack of facilities or suitable conditions, as in the case of pathogenic organisms for example. This has been the rule from the foundation of the Laboratory, and the range of research has consequently steadily increased with improvement of facilities. Changes of fashion have of course also occurred, and are reflected in the annual reports.

The policy has been to interest the strongest biologists and promising young investigators to bring their work to Woods Hole; and the degree of success of this policy has been the measure of success and influence of the Laboratory. The future of the Laboratory depends upon the continuance of this policy, and the elimination of conditions that tend to restrict its operation, whether these are based on inadequacy of equipment, administrative regulations, or community conditions. This is the most important policy of the Laboratory, if one may be allowed to rank essentials, for it ensures leadership and reputation. To supplement this policy the attendance of as many promising young investigators as possible should be encouraged.

If the number of investigators admitted is to be definitely restricted, and if the tendency towards an increase in numbers continues, it will be necessary to adopt more definite policies concerning admission of investigators than in the past. These should not, however, be of too binding a character, but rather a definition of principles within which the Director will have free scope for the exercise of his best judgment.

The established fees for research accommodations should be continued, and paid by the institution represented as far as possible. When this cannot be done it has been a frequent policy, more in the past than at present, to waive fees for distinguished investigators. Such arrangements have often been doubly blessed, in giving and in taking. The coöperation by institutions in the expenses of investigation of their representatives has been a strong stabilizing factor in the history of the Laboratory in more ways than one. This plan has never been more effective than at the present time, but it is important constantly to cultivate it.

The Committee recommends the continuance of our historical policy of maintaining courses of instruction. These should be contributory to research, and based upon the advantages of marine material, so that they are in no sense duplications of courses that may equally well be offered by universities. Of such courses there are several kinds. As contributory to research it is not meant that all necessarily lead directly to research as a final preparatory step, but that they may sometimes fill essential gaps in education for the kind of biological research intended by the individual. Preference for admission to courses should be given to students whose promise or declared intention indicates a professional career in the field of biology. Such students should, and do, derive great profit, not only from the actual instruction, but also from the scientific contacts that they make at Woods Hole.

The Trustees should maintain control of courses to see that proper content and principles of admission are preserved. The Executive Committee has for some time held a conference with the heads of courses each year with these purposes in mind.

Strict limitation of the numbers admitted to each course should be observed in the future as in the past. It should also be a policy to provide better and more stable laboratory accommodations.

F. Buildings, Equipmnt and Grounds

The first question is whether our holdings of real estate are adequate for the future. This can be answered substantially in the affirmative. We already have considerable undeveloped harbor frontage; we now own all the land on the block on which the original buildings of the Laboratory stood; in the block immediately north there is only one parcel of land on Center Street not now in our possession; and there is no immediate reason for attempting to complete our ownership of the remainder of the block. For residential purposes we still have unsold lots in the Gansett tract, and no subdivision whatever has been made of the 100 acres in the Devil's Lane Tract.

The second question concerns the buildings. Here three main needs present themselves.

In the first place, additional stack space for the library is needed. At the present rate of growth the stacks will be fully occupied in very few years. It is essential for the work of the Laboratory that this growth should be continued. Additional space can be provided by a wing to the east of the present library. It has been suggested that the present reading room might be utilized for additional stack space and the catalogue room be converted into a reading room with other necessary readjustments; other suggestions for temporary relief have also been offered. But at most only a short postponement would be afforded in such ways. The problem should be faced and estimates secured for building additional stack space.

The second main need is to replace the present wooden buildings with a fireproof building of solid construction. The work of the classes and investigators in the wooden buildings is seriously hampered by vibration, and the buildings do not lend themselves readily to modern installations. These buildings range in age from forty to fifty years, and they constitute a real fire hazard. This need should also receive the earnest attention of the Trustees.

Additional space is also needed for various technical services necessitated by the increasing complexity of important kinds of biological research in recent years, and which are not adequately provided for at present. Among these needs are those for space for autoclaves and sterilizers, which must now be used in rooms occupied by investigators, space for stills, which are now very disadvantageously housed in the boiler room in the basement of the Brick Building, additional shop space, particularly for use by investigators for relatively simple operations which they can carry out themselves, additional space for housing small animals, dehumidified and air-conditioned rooms, additional dark rooms, etc. Doubtless most of these needs could be cared for on the lower floors of the proposed addition for the Library. They ought, in any event, not to be forgotten. Furthermore, since needs of this sort are likely to increase in future years and are less predictable than the growth of the library, ample reserve space should be provided for them.

Our waterfront should be improved by landscaping and other ways so as to furnish a dignified frontage and water approach to the Laboratory. The George M. Gray Museum should have more adequate housing, and there are numerous other desirable small improvements that should be undertaken as soon as possible.

It is becoming increasingly important that the Supply Department be enabled to collect material for research from a wider area. To this end there should be a larger motor boat, and it is highly desirable that a resident naturalist be associated with the department who could study ecological conditions from year to year with a view to establishing sources of more abundant and more varied material for research. The standing Committee in the Supply Department should be asked to formulate the aims and policies of the Department.

G. Library

The Library Committee should be asked to formulate the aims and policies of the library.

H. Apparatus

Similarly, the Apparatus Committee should be asked to formulate its aims and policies.

I. Finances and Fiscal Policies

In 1932 the income from our endowment funds was $55,668, representing a return of 5 per cent on book value. It is now approximately $43,000, representing a return of 3.8 per cent on book value. The decrease in yield has been due partly to the necessity of refunding operations at lower interest rates; but the most drastic reductions in income have been suffered on the mortgage participations, some of which have been foreclosed, and others have had the interest rate much reduced.

The outstanding arrears of income amounted to $18,094 in 1935 but were reduced to $12,775 at the end of 1937. For three years the income has been supported by payment of arrears, a condition that cannot continue indefinitely.

With a loss of annual income from endowment amounting to over $12,000 there has been a considerable increase in attendance, which has not been compensated for by increased fees for research space. The cost of most apparatus and materials has recently risen appreciably and is likely to rise still farther. It is also certain that the progress of biological research will continually create new demands for special apparatus and equipment which must be met if the Laboratory is to retain its present position in scientific research. As a matter of fact, the budget of the Laboratory has been kept balanced since the beginning of the depression only by economies which have considerably handicapped the work of many of our investigators. Furthermore, although necessary upkeep has been maintained, certain desirable repairs to the buildings and equipment have been postponed since the early years of the depression but cannot be deferred much longer. Among the more expensive items that will require attention within the next few years are battery replacement, a new heating system for the Brick Building, repairs of the salt water system, painting and waterproofing of the brick buildings, etc. Reserves should also be built up to cover further depreciation of the buildings and equipment owned by the Laboratory, and to provide for the retirement of the Howes mortgage and for future purchases of property, etc. The problem of sewage disposal which may arise at any time is also likely to involve very considerable expense.

It is clear that substantial increase of the endowment of the Laboratory is necessary if we are to aim to restore the income to its pre-depression value, to provide adequately for the upkeep of the present plant, for the establishment of necessary reserves, and to meet increasing costs of operation.

As a partial offset to the loss of endowment income since 1931, the dividends of the General Biological Supply House increased from $2,032 in 1931 to $12,700 in 1936. The income from fees of students and investigators cannot be increased much unless considerably higher rates are established, which seems undesirable.

The Committee agrees that the most important fiscal policies to pursue are first to increase endowment and second to establish cash reserves for depreciation and contingencies. It is fair to point out in the latter connection that cash reserves previously accumulated have been used for purchase of real estate and that considerable sums have also gone each year into capital improvements. The Committee recommends that additional endowments secured be placed in the same trusts as the present major endowment funds, or in another trust under the same principles.

J. Community Arrangements and Responsibilities

As our community has grown and assumed a more settled character, community needs have increased. The primitive needs of food and lodging have from the beginning been recognized as an official responsibility of the Laboratory; the present arrangements for low-cost housing do not appear to be entirely adequate for a community of our size. Their administration should be in the hands of the Business Manager subject to control by the superior administrative officers. An advisory committee is not recommended.

For those who desire to own their own homes, the Laboratory possesses ample real estate in the Gansett and Devil's Lane tracts, saleable to members at reasonable rates and terms. The acquisition of these tracts has aided to prevent unreasonable increase of price of village properties.

These provisions should be regarded as terminating the direct and exclusive official responsibility of the Laboratory for community purposes. While the Laboratory should aid in securing recreational facilities, the responsibility for operating them should be in the hands of the community itself. This principle has operated well in the case of the " M. B. L. Club " and the " M. B. L. Tennis Club." The Laboratory has furnished land and buildings, and from time to time has made loans for improvements, and it may yet appear desirable to provide an addition to the building of the M. B. L. Club. But these organizations should operate under their own membership and fees. With the acquisition of the bathing beach the question arises whether the same principles could not be made to operate there.

K. Summary of Principal Recommendations

1. That the Marine Biological Laboratory pursue a policy of consolidation rather than expansion for the present.
2. That in pursuit of this policy steps be taken to provide the following major improvements:

    a. Secure additional funds for endowment.
    b. Provide additional stack room to accommodate approximately 100,000 additional volumes, together with study cubicles.
    c. Replace present wooden laboratories by a building, or buildings, of stable fireproof construction, providing an intermediate court to set off the present main building.

   d. In connection with the library construction provide adequate space for expansion of various technical services as described in II F.
   e. Make provision for the series of miscellaneous needs enumerated in the body of the report.

3. Maintain the principles of coöperation (II B.), organization and government (II C.), administration (II D.), research and instruction, (II E.), that have served so well in the past, as the basis for future development.
4. Additional endowment funds as received should be placed, like the present main endowment funds, in trust. Reserves for depreciation, contingencies, improvements and retirement fund should be set up out of income (II I.).
5. Responsibility for recreational facilities should be placed as far as possible on voluntary organizations within our scientific community (II J.).

2. THE STAFF, 1938

Charles Packard, Associate Director, Assistant Professor of Zoölogy, Institute of Cancer Research, Columbia University.

ZOÖLOGY

I. Investigation

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
E. G. Conklin, Professor of Zoölogy, Princeton University.
Caswell Grave, Professor of Zoölogy, Washington University.
H. S. Jennings, Professor of Zoölogy, Johns Hopkins University.
Frank R. Lillie, Professor of Embryology Emeritus, The University of Chicago.
C. E. McClung, Professor of Zoölogy, University of Pennsylvania.
S. O. Mast, Professor of Zoölogy, Johns Hopkins University.
T. H. Morgan, Director of the Biological Laboratory, California Institute of Technology.
G. H. Parker, Professor of Zoölogy Emeritus, Harvard University.
E. B. Wilson, Professor of Zoölogy Emeritus, Columbia University.
Lorande L. Woodruff, Professor of Protozoölogy, Yale University.

II. Instruction

T. H. Bissonnette, Professor of Biology, Trinity College.
P. S. Crowell, Jr., Instructor in Zoölogy, Miami University.
C. E. Hadley, Associate Professor of Biology, New Jersey State Teachers College at Montclair.
F. R. Kille, Assistant Professor of Zoölogy, Swarthmore College.
A. M. Lucas, Associate Professor of Zoölogy, Iowa State College.
S. A. Matthews, Assistant Professor of Biology, Williams College.
A. J. Waterman, Assistant Professor of Biology, Williams College.

BIOLOGICAL LABORATORY LIBRARY WOODS HOLE, MASS.

Junior Instructors

W. F. Hahnert, Associate Professor of Zoölogy, Ohio Wesleyan University.

J. S. Rankin, Teaching Fellow in Zoölogy, Amherst College.

PROTOZOÖLOGY

I. Investigation

(*See Zoölogy*)

II. Instruction

Gary N. Calkins, Professor of Protozoölogy, Columbia University.

G. W. Kidder, Assistant Professor of Biology, Brown University.

Elizabeth Drumtra Hughes, Lecturer in Zoölogy, Barnard College.

EMBRYOLOGY

I. Investigation

(*See Zoölogy*)

II. Instruction

W. W. Ballard, Assistant Professor of Biology and Anatomy, Dartmouth College.

Hubert B. Goodrich, Professor of Biology, Wesleyan University.

Viktor Hamburger, Assistant Professor of Zoölogy, Washington University.

Oscar Schotté, Assistant Professor of Biology, Amherst College.

Douglas M. Whitaker, Professor of Zoölogy, Stanford University.

PHYSIOLOGY

I. Investigation

William R. Amberson, Professor of Physiology, University of Maryland, School of Medicine.

Harold C. Bradley, Professor of Physiological Chemistry, University of Wisconsin.

Walter E. Garrey, Professor of Physiology, Vanderbilt University Medical School.

M. H. Jacobs, Professor of General Physiology, University of Pennsylvania.

Ralph S. Lillie, Professor of General Physiology, The University of Chicago.

Albert P. Mathews, Professor of Biochemistry, University of Cincinnati.

II. Instruction

Teaching Staff

Laurence Irving, Professor of Biology, Swarthmore College.

Robert Chambers, Professor of Biology, New York University.

J. K. W. Ferguson, Assistant Professor of Physiology, Ohio State University.
Kenneth C. Fisher, Assistant Professor of Experimental Biology, University of Toronto.
C. Ladd Prosser, Assistant Professor of Physiology, Clark University.
Carl F. Schmidt, Professor of Pharmacology, University of Pennsylvania.
F. J. M. Sichel, Instructor in Physiology, University of Vermont, College of Medicine.

BOTANY

I. Investigation

C. E. Allen, Professor of Botany, University of Wisconsin.
S. C. Brooks, Professor of Zoölogy, University of California.
B. M. Duggar, Professor of Physiological and Economic Botany, University of Wisconsin.
Ivey F. Lewis, Professor of Biology, University of Virginia.
Wm. J. Robbins, Professor of Botany, University of Missouri.

II. Instruction

William Randolph Taylor, Professor of Botany, University of Michigan.
Francis Drouet, Research Fellow, Yale University.
B. F. D. Runk, Research Fellow, University of Virginia.

GENERAL OFFICE

F. M. MacNaught, Business Manager.
Polly L. Crowell, Assistant.
Edith Billings, Secretary.

RESEARCH SERVICE AND GENERAL MAINTENANCE

Samuel E. Pond, Technical Mgr.
G. Failla, X-ray Physicist.
T. E. Larkin, Superintendent.
W. C. Hemenway, Carpenter.
Lester F. Boss, Technician.
J. D. Graham, Glassblower.
J. T. Simonton, Assistant.
Elbert P. Little, X-ray.

LIBRARY

Priscilla B. Montgomery (Mrs. Thomas H. Montgomery, Jr.), Librarian.
Deborah Lawrence, Secretary.
Mary A. Rohan, S. Mabell Thombs, Assistants.

SUPPLY DEPARTMENT

James McInnis, Manager.
Milton B. Gray, Collector.
A. M. Hilton, Collector.
A. W. Leathers, Shipping Dept.
Geoffrey Lehy, Collector.
Walter Kahler, Collector.
F. N. Whitman, Collector.
Ruth S. Crowell, Secretary.
Grace Harman, Secretary.

THE GEORGE M. GRAY MUSEUM

George M. Gray, Curator Emeritus.

3. INVESTIGATORS AND STUDENTS, 1938

Independent Investigators

Abramowitz, A. A., Research Assistant, Harvard University.
Adams, Mark H., Fellowship in Pneumonia Research, Rockefeller Institute for Medical Research.
Amberson, William R., Professor of Physiology, University of Maryland, School of Medicine.
Anderson, R. L., Professor of Biology, Johnson C. Smith University.
Anderson, Rubert S., Research Associate, Princeton University.
Angerer, C. A., Instructor, University of Pennsylvania.
Appel, Frederick W., Associate Professor of Biology, St. John's College.
Armstrong, Philip B., Professor of Anatomy, University of Alabama, School of Medicine.
Ballard, William W., Assistant Professor in Zoölogy and Anatomy, Dartmouth College.
Barth, Lester G., Assistant Professor of Zoölogy, Columbia University.
Beck, Lyle V., Research Fellow, University of Pennsylvania, School of Medicine.
Bedichek, Sarah, Associate Professor of Biology, North Texas Agricultural College.
Bernstein, Felix, Professor of Biometrics, New York University, College of Medicine.
Bertalanffy, Ludwig von, Privatdozent an der Universitat Wien, Wien, Germany.
Bissonnette, T. H., Professor and Head of Biology Department, Trinity College.
Black, Lindsay MacLeod, Assistant, Rockefeller Institute for Medical Research.
Boche, Robert D., Research Assistant, Department of Embryology, Carnegie Institution of Washington.
Bodian, David, National Research Council Fellow in Medicine, University of Michigan.
Boettiger, Edward G., Graduate Student, Harvard University.
Boernstein, Walter, Honorary Research Fellow, Yale University, School of Medicine.
Botsford, E. Frances, Assistant Professor of Zoölogy, Connecticut College.
Bozler, Emil, Assistant Professor of Physiology, Ohio State University.
Bradley, Harold C., Professor of Physiological Chemistry, University of Wisconsin.
Brambel, Charles E., Instructor in Zoölogy, Johns Hopkins University.
Bronfenbrenner, J. J., Professor of Bacteriology and Immunology, Washington University, School of Medicine.
Buck, John B., Research Assistant, Department of Embryology, Carnegie Institution of Washington.
Budington, Robert A., Professor of Zoölogy, Oberlin College.
Burton, Alan C., Fellow in Medical Physics, Johnson Foundation, University of Pennsylvania.
Cable, Raymond M., Assistant Professor of Parasitology, Purdue University.
Calkins, Gary N., Professor of Protozoölogy, Columbia University.
Carothers, E. Eleanor, Research Associate, University of Iowa.
Carpenter, Russell L., Assistant Professor of Anatomy, College of Physicians and Surgeons, Columbia University.
Chambers, Robert, Research Professor of Biology, Washington Square College, New York University.

Cheney, Ralph H., Chairman of Biology Department, Professor of Biology, Long Island University.
Claff, C. Lloyd, 5 Van Beal Road, Randolph, Massachusetts.
Clark, Eleanor L., Department of Anatomy, University of Pennsylvania, School of Medicine.
Clark, Eliot R., Professor of Anatomy, University of Pennsylvania, School of Medicine.
Claude, Albert, Associate, Rockefeller Institute for Medical Research.
Clowes, G. H. A., Director of Research, Lilly Research Laboratories.
Cole, Elbert C., Professor of Biology, Williams College.
Cole, Kenneth S., Associate Professor of Physiology, College of Physicians and Surgeons, Columbia University.
Colwin, Arthur L., Research Fellow, Osborn Zoölogical Laboratory, Yale University.
Commoner, Barry, Graduate Student, Harvard University.
Conklin, Edwin G., Professor Emeritus of Biology, Princeton University.
Cooper, Kenneth W., Lydig Fellow, Columbia University.
Copeland, D. Eugene, Assistant, Harvard University.
Copeland, Manton, Professor of Biology, Bowdoin College.
Corson, Samuel A., Instructor, Cell Physiology, Division of General Education, Washington Square College, New York University.
Costello, Donald P., Assistant Professor of Zoölogy, University of North Carolina.
Cowles, Rheinart P., Professor of Zoölogy, Johns Hopkins University.
Cox, Edward H., Professor of Chemistry, Swarthmore College.
Croasdale, Hannah T., Technical Assistant in Zoölogy, Dartmouth College.
Crouse, Helen V., Research Assistant, Carnegie Institution of Washington.
Crowell, Prince S., Jr., Instructor in Zoölogy, Miami University.
Curtis, W. C., Professor of Zoölogy, University of Missouri.
Denny, Martha, Instructor, Connecticut College.
Diller, Irene Corey, Research Associate in Zoölogy, University of Pennsylvania.
Diller, William F., Assistant Professor of Zoölogy, Dartmouth College.
Drouet, Francis, Theresa Seessel Research Fellow, Yale University.
Duryee, William R., Research Associate in Biology, Washington Square College, New York University.
Elftman, Herbert, Assistant Professor of Zoölogy, Columbia University.
Elwyn, Adolph, Associate Professor of Neurology, College of Physicians and Surgeons, Columbia University.
Failla, G., Physicist, Memorial Hospital, New York City.
Fennell, Richard A., Instructor in Zoölogy, Michigan State College.
Ferguson, J. K. W., Assistant Professor of Physiology, Ohio State University.
Fisher, Kenneth C., Assistant Professor of Experimental Biology, University of Toronto.
Florkin, Marcel, Professor of Biochemistry, University of Liège, Belgium.
Forbes, Henry S., Associate in Neuropathology, Harvard Medical School.
Fries, E. F., Assistant Professor, College of the City of New York.
Frisch, John A., Professor of Biology, Canisius College.
Fry, Henry J., Visiting Investigator, Cornell University Medical College.
Furth, Jacob, Assistant Professor of Pathology, Cornell University Medical College.
Garrey, Walter E., Professor of Physiology, Vanderbilt University, School of Medicine.
Geldard, Frank A., Professor of Psychology, University of Virginia.
Gilman, Mr. Lauren C., Laboratory Instructor in Biology, Johns Hopkins University.
Glaser, Otto, Professor of Biology, Amherst College.

Goodrich, H. B., Professor of Biology, Wesleyan University.
Gordon-Königes, Helmut, Fellow, Rockefeller Foundation.
Grabar, Pierre, Fellow, Rockefeller Foundation, Chef de Laboratoire a l'Institut Pasteur, Paris, France.
Grant, Ronald, Lecturer in Physiology, McGill University.
Grave, Caswell, Professor of Zoölogy, Washington University.
Gray, Peter, Lecturer in Vertebrate Embryology, Edinburgh University.
Guthrie, Mary J., Associate Professor of Zoölogy, University of Missouri.
Hadley, Charles E., Associate Professor of Biology, Montclair State Teachers' College.
Hahnert, William F., Associate Professor of Zoölogy, Ohio Wesleyan University.
Hamburger, Viktor, Assistant Professor, Washington University.
Harris, Daniel L., Instructor, University of Pennsylvania.
Harrold, C. M., Graduate Assistant, New York University.
Hartman, Frank A., Chairman and Professor of Physiology, Ohio State University.
Harvey, Ethel Browne, Investigator, Princeton University.
Harvey, E. Newton, Professor of Physiology, Princeton University.
Heilbrunn, L. V., Associate Professor of Zoölogy, University of Pennsylvania.
Henshaw, Paul S., Biophysicist, Memorial Hospital, New York City.
Hetzer, H. O., Associate Animal Husbandman, United States Department of Agriculture, Washington, D. C.
Hickson, Anna Keltch, Research Chemist, Lilly Research Laboratories.
Hiestand, William A., Associate Professor of Physiology, Purdue University.
Hill, Edgar S., Instructor in Biochemistry, Washington University.
Hill, Samuel E., Assistant in General Physiology, Rockefeller Institute for Medical Research.
Hodge, Charles, 4th, Assistant Professor, Temple University.
Hodgkin, Alan L., Demonstrator in Physiology, Cambridge, England.
Hopkins, Dwight L., Research Assistant, Johns Hopkins University.
Howe, H. E., Editor, Industrial and Engineering Chemistry, Washington, D. C.
Hughes, Elizabeth Drumtra, Lecturer in Zoölogy, Barnard College.
Hughes, Roscoe D., Assistant in Zoölogy, Columbia University.
Hunninen, Arne V., Professor of Biology, Oklahoma City University.
Hunter, George W., III, Assistant Professor of Biology, Wesleyan University.
Hunter, Laura N., Assistant Professor, Pennsylvania College for Women.
Hutchings, Lois M., Teacher of Biology, Weequahic High School, Newark, N. J.
Irving, Laurence, Professor of Biology, Swarthmore College.
Jacobs, M. H., Professor of General Physiology, University of Pennsylvania.
Jeffers, Katharine R., Instructor in Zoölogy, Duke University.
Jenkins, George B., Professor of Anatomy, George Washington University.
Johlin, J. M., Associate Professor of Biochemistry, Vanderbilt University, School of Medicine.
Jones, E. Ruffin, Jr., Associate Professor, College of William and Mary.
Jones, Ruth McClung, Instructor in Botany and Zoölogy, Swarthmore College.
Karady, Stephen, Assistant Professor, Internal Clinic of the Francis Joseph University, Hungary.
Kidder, George W., Assistant Professor of Biology, Brown University.
Kiese, Manfred, Rockefeller Fellow, Assistant, Pharmacological Institute of the University of Berlin.
Kille, Frank R., Assistant Professor of Zoölogy, Swarthmore College.
Kindred, J. E., Associate Professor of Histology and Embryology, University of Virginia.
Knowlton, Frank P., Professor of Physiology, Syracuse University, College of Medicine.
Kopac, M. J., Research Associate, Washington Square College, New York University.

Korr, Irvin M., Instructor in Physiology, New York University, College of Medicine.
Krahl, M. E., Research Chemist, Lilly Research Laboratories.
Kreezer, George, Assistant Professor of Psychology, Cornell University.
Krieg, Wendell J. S., Instructor in Anatomy, New York University, College of Medicine.
Kunitz, Moses, Associate, Rockefeller Institute for Medical Research.
Lancefield, Donald E., Associate Professor in Biology, Queens College.
Levy, Milton, Assistant Professor in Chemistry, New York University, College of Medicine.
Liebmann, Emil, Fisheries Service of the British Government in the Near East.
Lillie, Frank R., Professor of Embryology, Emeritus, The University of Chicago.
Lillie, Ralph S., Professor of General Physiology, The University of Chicago.
Loeb, Leo, Professor Emeritus of Pathology, Washington University, School of Medicine.
Lucas, Alfred M., Associate Professor of Zoölogy, Iowa State College.
Lucas, Miriam Scott, Iowa State College.
Ludwig, Daniel, Associate Professor of Biology, New York University.
Lynn, W. Gardner, Instructor, Johns Hopkins University.
McCann, Lewis P., Graduate Assistant, University of Maryland.
McClung, C. E., Director Zoölogical Laboratory, University of Pennsylvania.
McCurdy, Mary Derrickson, Graduate Student, Duke University.
McCurdy, Harold G., Research Assistant, Duke University.
MacDougall, Mary Stuart, Head of Biology Department, Agnes Scott College.
McFarland, Elsie Laity, Instructor in Zoölogy, Wheaton College.
MacLennan, Ronald F., Associate Professor of Zoölogy, State College of Washington.
Magruder, Samuel R., Assistant in Anatomy, Cornell University Medical College.
Maloeuf, N. S. Royston, Honorary Research Fellow, Yale University.
Martin, W. E., Assistant Professor of Zoölogy, DePauw University.
Mast, S. O., Professor of Zoölogy in Charge of General Physiology, Johns Hopkins University.
Mathews, Albert P., Andrew Carnegie Professor of Biochemistry, University of Cincinnati.
Matthews, Samuel A., Assistant Professor of Biology, Williams College.
Mavor, James W., Professor of Biology, Union College.
Miller, James A., Instructor in Anatomy, University of Michigan.
Molter, John A., Instructor, University of Notre Dame.
Morgan, Lilian V., Pasadena, California.
Morgan, T. H., Professor of Biology, California Institute of Technology.
Morrill, Charles V., Associate Professor of Anatomy, Cornell University Medical College.
Muller, H. J., Institute of Animal Genetics, University of Edinburgh.
Navez, Albert E., Instructor in Biology, Milton Academy.
Newton, William H., Reader in Physiology, Institute of Physiology, University College, London, England.
Nonidez, José F., Professor of Anatomy, Cornell University Medical College.
Northrop, John H., Member, Rockefeller Institute for Medical Research.
Obreshkove, Vasil, Professor of Biology, Bard College, Columbia University.
O'Brien, John P., Johns Hopkins University.
Olson, Magnus, Instructor in Zoölogy, University of Minnesota.
Orr, Paul R., Assistant Professor, Brooklyn College.
Oster, Robert H., Assistant Professor, University of Maryland, School of Medicine.
Osterhout, W. J. V., Member, Rockefeller Institute for Medical Research.
Packard, Charles, Assistant Professor of Zoölogy, Institute of Cancer Research, Columbia University.

Parker, G. H., Professor of Zoölogy Emeritus, Harvard University.
Parmenter, Charles L., Associate Professor, University of Pennsylvania.
Parpart, Arthur K., Associate Professor, Princeton University.
Patrick, Ruth, Associate Curator of Department of Microscopy, Academy of the Natural Sciences of Philadelphia.
Pierce, Madelene, Vassar College.
Pierson, Bernice F., Graduate Student, Johns Hopkins University.
Pipkin, C. A., University of Texas.
Plough, Harold H., Professor of Biology, Amherst College.
Pollister, Arthur W., Assistant Professor of Zoölogy, Columbia University.
Pond, Samuel E., Technical Manager, Marine Biological Laboratory.
Prosser, C. Ladd, Assistant Professor of Physiology, Clark University.
Rabinowitch, E., Research Associate, University College, London, England.
Rankin, John S., Jr., Teaching Fellow in Biology, Amherst College.
Root, Raymond W., Assistant Professor of Biology, College of the City of New York.
Rous, Peyton, Member in Pathology and Bacteriology, Rockefeller Institute for Medical Research.
Rugh, Roberts, Instructor in Zoölogy, Hunter College.
Runk, B. F. D., Research Fellow, University of Virginia.
Russell, Alice Mary, Instructor in Zoölogy, University of Pennsylvania.
Sabin, Albert B., Associate, Pathology and Bacteriology, Rockefeller Institute for Medical Research.
Sandow, Alexander, Assistant Professor of Biology, Washington Square College, New York University.
Saslow, George, Instructor in Physiology, Harvard School of Public Health.
Sayles, Leonard P., Assistant Professor of Biology, College of the City of New York.
Schaeffer, Asa A., Professor of Biology, Temple University.
Schechter, Victor, Instructor, College of the City of New York.
Schmidt, Carl F., Professor of Pharmacology, University of Pennsylvania.
Schmidt, Ida Genther, Assistant Professor of Anatomy, University of Cincinnati, College of Medicine.
Schmidt, L. H., Research Fellow, Christ Hospital and University of Cincinnati, College of Medicine.
Schotté, Oscar E., Associate Professor of Biology, Amherst College.
Scott, Allan C., Assistant Professor of Biology, Union College.
Scott, Sister Florence Marie, Professor of Zoölogy, Seton Hill College.
Shaw, Myrtle, Senior Bacteriologist, New York State Department of Health.
Sichel, Elsa Keil, Assistant Professor of Zoölogy, Rutgers University.
Sichel, F. J. M., Instructor in Physiology, University of Vermont, College of Medicine.
Slifer, Eleanor H., Assistant Professor, State University of Iowa.
Smith, Dietrich C., Associate Professor of Physiology, University of Maryland, School of Medicine.
Smith, Jay A., Instructor in Biology, Johns Hopkins University.
Smith, Marshall E., Student, Johns Hopkins University, Medical School.
Solberg, Archie N., Instructor in Biology, University of Toledo.
Southwick, Mildred D., Ecologist, Department of Botany, Vassar College.
Speidel, Carl C., Professor of Anatomy, University of Virginia, Medical School.
Stanley, W. M., Associate Member, Rockefeller Institute for Medical Research.
Stannard, J. Newell, Instructor in Physiology, University of Rochester, Medical School.
Steinbach, H. Burr, Assistant Professor of Zoölogy, Columbia University.
Steven, David M., Magdalen College, Oxford, England.
Steinhardt, Jacinto, Research Fellow, Rockefeller Foundation, Harvard Medical School.

Stockard, Charles R., Professor of Anatomy, Cornell University Medical College.
Stokey, Alma G., Professor of Botany, Mount Holyoke College.
Taylor, Wm. Randolph, Professor of Botany, University of Michigan.
Thornton, Charles S., Assistant Professor of Biology, Kenyon College.
Townsend, Grace, Professor, Great Falls Normal College.
Trombetta, Vivian V., Assistant in Botany, Barnard College, Columbia University.
Turner, C. L., Professor of Zoölogy, Northwestern University.
Turner, John P., Assistant Professor of Zoölogy, University of Minnesota.
Uhlenhuth, Eduard, Professor of Anatomy, University of Maryland, School of Medicine.
Vandebroek, Georges, Assistant in the Laboratory of Embryology and Histology, Faculty of Medicine, University of Ghent, Belgium.
Vicari, Emelia M., Associate in Anatomy, Cornell University Medical College.
Visscher, J. Paul, Professor of Biology, Western Reserve University.
Walzl, Edward M., Instructor, Johns Hopkins University, School of Medicine.
Waterman, A. J., Assistant Professor of Biology, Williams College.
Weiss, Paul, Associate Professor, The University of Chicago.
Wenrich, D. H., Professor of Zoölogy, University of Pennsylvania.
Whitaker, D. M., Professor of Biology, Stanford University.
White, Michael J. D., Lecturer in Zoölogy, University College, London, England.
White, Thomas N., Jr., Assistant Biophysicist, United States Public Health Service, National Institute of Health.
Whiting, Anna R., Guest Research Investigator, University of Pennsylvania.
Whiting, P. W., Associate Professor of Zoölogy, University of Pennsylvania.
Wichterman, Ralph, Instructor, Temple University.
Wieman, H. L., Professor of Zoölogy, University of Cincinnati.
Wiersma, Cornelis A. G., Associate Professor of Physiology, California Institute of Technology.
Wilhelmi, Raymond W., Graduate Assistant, New York University.
Willey, Charles H., Assistant Professor of Biology, New York University.
Willier, Benjamin H., Chairman, Division of Biological Sciences, University of Rochester.
Wilson, Edmund B., Professor Emeritus in Residence, Columbia University.
Wolf, E. Alfred, Associate Professor of Biology, University of Pittsburgh.
Wolf, Opal M., Assistant Professor of Biology, Goucher College.
Woodruff, L. L., Professor of Protozoölogy, Yale University.
Yancey, P. H., Chairman, Department of Biology, Spring Hill College.
Young, Roger A., Graduate Student, University of Pennsylvania.

Beginning Investigators

Algire, Glenn H., Weaver Research Fellow in Anatomy, University of Maryland.
Arena, Julio F. de la, Auxiliary Professor of Biology, Universidad de la Habana.
Ballentine, Robert, Graduate Student, Princeton University.
Belcher, Jane C., Graduate Assistant in Zoölogy, University of Missouri.
Belda, Walter H., Graduate Student in Zoölogy, Johns Hopkins University.
Bishop, David W., Instructor, University of Pennsylvania.
Bliss, Alfred F., Laboratory Assistant, Department of Biophysics, Columbia University.
Brill, Edmund R., Graduate Student in Biology, Harvard University.
Castle, Ruth M., Assistant in Zoölogy, Vassar College.
Churney, Leon, Instructor in Zoölogy, University of Pennsylvania.
Cooper, Ruth Snyder, Assistant in Zoölogy, Columbia University.
Cornman, Ivor, Teaching Fellow, Washington Square College, New York University.
Crowell, Villa Bailey, Miami University.

Donnellon, J. A., Graduate Student, University of Pennsylvania.
Ferguson, Frederick P., Undergraduate Assistant, Wesleyan University.
Frank, John A., Medical Student, Yale University.
Glancy, Ethel, Teaching Fellow, Washington Square College, New York University.
Goldin, Abraham, Graduate Student, Columbia University.
Grave, Caswell, II, Assistant, Washington University.
Guttman, Rita, Graduate Student in Physiology, College of Physicians and Surgeons, Columbia University.
Hall, Thomas S., Graduate Student, Yale University.
Hiatt, Edwin P., Research Fellow, University of Maryland, School of Medicine.
Hinchey, M. Catherine, Graduate Student, University of Pennsylvania.
Hobson, Lawrence B., Graduate Assistant in Zoölogy, University of Cincinnati.
Hollingsworth, Josephine, Graduate Student, University of Pennsylvania.
Hutchins, Louis W., Graduate Student, Yale University.
Kriete, Bertrand C., Graduate Assistant in Zoölogy, University of Cincinnati.
Lambert, Barbara, Graduate Assistant in Physiology, Mount Holyoke College.
Levenson, Alfred S., Graduate Student, University of Pittsburgh.
Lipman, Harry J., Graduate Assistant, University of Pittsburgh.
Ludwig, Francis W., Graduate Student, University of Pennsylvania.
Mayo, Mercedes, Assistant Professor of Biology, Universidad de la Habana.
Moore, Anna Betty Clark, Graduate Student, Columbia University.
Moore, John A., Assistant in Zoölogy, Columbia University.
Mullins, Lorin J., Graduate Student, University of California.
Ramsey, Helen J., Purdue University.
Ray, D. T., Assistant Professor of Biology, Johnson C. Smith University.
Rose, S. Meryl, Assistant in Zoölogy, Columbia University.
Ryan, Francis J., Assistant in Zoölogy, Columbia University.
Schenthal, Joseph E., Weaver Fellow in Anatomy, University of Maryland, School of Medicine.
Schoenborn, Henry W., Graduate Assistant, New York University.
Schoepfle, G. M., Research Assistant, Princeton University.
Shaver, John R., Museum Assistant, University of Pennsylvania.
Silber, Robert H., Assistant and Graduate Student, Washington University.
Singer, Marcus, Student Worker, University of Pittsburgh.
Smith, Audrey U., Assistant in Physiology, Vassar College.
Stewart, Brooks, Graduate Student, University of Pennsylvania.
Von Dach, Herman, Assistant, Ohio State University.
Weinberg, Victor, The University of Chicago.
White, Elizabeth C., Student, University of Pennsylvania.
Wilbur, Karl M., Harrison Fellow, University of Pennsylvania.
Wise, John S., Medical Student, University of Pennsylvania, School of Medicine.
Zwilling, Edgar, Assistant, Columbia University.

Research Assistants

Alley, Armine, Research Assistant, McGill University.
Anderson, Katherine, Research Technician, Vanderbilt University.
Armstrong, Charles W. J., Demonstrator in Biology, University of Toronto.
Armstrong, Louise S., Research Assistant, University of Alabama.
Auringer, Jack, Research Assistant, Columbia University.
Baker, Linville A., Lilly Research Laboratories.
Beck, Naomi, Graduate Student, The University of Chicago.
Bender, Joseph C., Research Assistant, Swarthmore College.
Bernstein, Marianne, 325 E. 41st Street, New York City.
Bertalanffy, Maria M. von, Universitat Wien, Wien, Germany.

Bien, Bettina H., Wheaton College.
Birnbaum, Sanford M., University Scholar, University of Cincinnati.
Birnbaum, William F., Research Assistant, New York University, College of Medicine.
Black, Edgar C., Research Associate, Swarthmore College.
Bowen, William J., Bruce Fellow, Johns Hopkins University.
Brownell, Katharine A., Research Assistant, Ohio State University.
Burnett, Jack M., Graduate Student, Washington University.
Cecil, Sam, Assistant, Vanderbilt University, School of Medicine.
Chambers, Edward L., Research Assistant, New York University.
Cohen, Irving, Memorial Hospital, New York City.
Costello, Helen Miller, University of North Carolina.
Crawford, John D., Milton Academy, Milton, Massachusetts.
Curtis, Howard J., Associate in Physiology, College of Physicians and Surgeons, Columbia University.
Dienes, Priscilla, 27 Walker Street, Cambridge, Massachusetts.
Dowding, Grace L., Research Technician, University of Maryland, School of Medicine.
Downs, J. Hunter, Undergraduate, Colgate University.
Dugal, Louis-Paul, Instructor in Biology, University of Montreal.
Dumm, Mary E., 13 Sampson Avenue, Madison, New Jersey.
Dziemian, Arthur J., Graduate Student, Princeton University.
Evans, Hiram J., Assistant in Biology, Williams College.
Fink, Harold K., Student, Princeton University.
Finkel, Asher J., Research Assistant, The University of Chicago.
Foster, Richard, Milton Academy, Milton, Massachusetts.
Fox, Ernest L., Research Assistant, Miami University.
Funkhouser, Elizabeth M. J., Swarthmore College.
Gettemanz, John F., Laboratory Assistant, Rockefeller Institute for Medical Research.
Grand, C. G., Research Associate, Washington Square College, New York University.
Hamdi, Turgut N., University of Pennsylvania.
Hatch, Cleora, Technician, Cornell University Medical College.
Horn, Edward C., Assistant, Trinity College.
Howell, Charles D., Professor of Biology, Elizabethtown College.
Hutchens, John, Lilly Research Laboratories.
Keefe, Eugene L., Research Assistant, Washington University.
Kemp, Emily J., Instructor in Physiology, University of Maryland, School of Medicine.
Levin, Louis, Student, University of Cincinnati, College of Medicine.
Lewis, Lena, Research Assistant in Physiology, Ohio State University.
Linscheid, Martha, Research Assistant, Western Reserve University.
Lyon, Rhea C., Research Technician, University of Maryland, School of Medicine.
McDonald, Margaret Ritchie, Senior Technician, Rockefeller Institute for Medical Research.
Martin, Mary S., University of Rochester, School of Medicine.
Martin, Rosemary D. C., Assistant, University of Toronto.
Melland, Amicia M., Research Worker, Carnegie Institution of Washington.
Milford, John J., Student, New York University.
Musser, Ruth E., Student, Goucher College.
Naumann, Rudolph V., Fellow in Physiology, New York University, College of Medicine.
Netsky, Martin, Research Assistant, University of Pennsylvania.
Norris, Charles H., Graduate Student, Princeton University.
Osborn, Clinton M., Research Fellow, Harvard University.

Pratt, David M., Student, Williams College.
Rawles, Mary E., Research Assistant, University of Rochester.
Safford, Virginia, Assistant, Swarthmore College.
Salzburg, Frederick P., Research Assistant, University of Minnesota.
Sawyer, Elizabeth L., Associate Professor of Biology, Converse College.
Scholl, Samuel M., Research Assistant, University of Toledo.
Selverstone, Bertram, Student, Harvard Medical School.
Simmons, Eric L., Research Assistant, Swarthmore College.
Sisson, Warren R., Jr., Assistant, Milton Academy.
Smith, Carl C., Iglauer Fellow in Biochemistry, University of Cincinnati.
Sneider, Elizabeth, Arnold Biological Fellow, Brown University.
Spencer, Joseph M., Research Assistant, College of Physicians and Surgeons, Columbia University.
Stenger, Albert H., Technician, New York University.
Stocker, Gail, Research Assistant, University of Pennsylvania.
Strickland, J. C., Graduate Instructor, University of Richmond.
Suddath, E. E., Technician, Washington University.
Taneri, Bedia, Graduate Student, New York University, College of Medicine.
Thompson, Raymond K., Research Assistant, University of Maryland.
tum Suden, Caroline, Research Fellow in Physiology, Boston University, School of Medicine.
Wagner, Carroll E., Research Technician, University of Maryland.
Wightman, John C., Assistant in Biology, Brown University.
Wilson, John Woodrow, Graduate Assistant in Zoölogy, Duke University.
Young, Saul B., Technician, Rockefeller Institute for Medical Research.

Students

BOTANY

Bader, Joan E., Montclair State Teachers College.
Bien, Bettina H., Student, Wheaton College.
Bonner, John T., Student, Harvard University.
Fender, Flora S., Preparator, University of Pennsylvania.
Graves, E. Irene, Senior Instructor in Biology, Bridgewater State Teachers College.
Hoffman, Elizabeth D., Mount Holyoke College.
Markle, Jane C., Smith College.
Postel, Frances H., Wellesley College.
Rutledge, Alma W., Graduate Student, Johns Hopkins University.
Schallek, William B., Harvard University.
Siegel, Marion T., New Jersey College for Women.
Ward, Henry S., Jr., Alabama Polytechnic Institute.

EMBRYOLOGY

Alley, Armine, Research Assistant, McGill University.
Armstrong, Florence H., Student, Dalhousie University.
Berry, Clyde, Jr., Washington University.
Blanchard, Joseph, Student, Wesleyan University.
Bookhout, Cazlyn G., Instructor in Zoölogy, Duke University.
Brush, Helen V., Vassar College.
Collier, Jane G., Assistant, University of Missouri.
Coppola, Armando R., Brothers College of Drew University.
Dobler, Marian, Goucher College.
Drury, Horace F., Harvard University.

Dunham, Donald W., Assistant in Zoölogy, Ohio State University.
Edds, Mac Vincent, Jr., Amherst College.
Fink, Harold K., Graduate Student, Princeton University.
Finkel, Asher J., Research Assistant, The University of Chicago.
Harrold, Charles M., Jr., Graduate Assistant, New York University.
Klein, Ethel L., University of Rochester.
Kurtz, Elizabeth L., Wilson College.
Lewisohn, Marjorie G., University of Michigan.
Milne, Walter S., Graduate Assistant, University of Missouri.
Philips, Frederick S., Graduate Assistant, University of Rochester.
Rogick, Mary D., Professor of Biology, College of New Rochelle.
Rogoff, William M., Graduate Student, Yale University.
Rothermel, Julia E., Professor of Biology, Western College.
Soderwall, Arnold L., Assistant in Zoölogy, University of Illinois.
Spangler, Juliet M., Wheaton College.
Stableford, Louis T., Laboratory Assistant, Yale University.
Stevens, Florence F., New Jersey College for Women.
Taylor, Harriett E., Radcliffe College.
Terzian, Annette V., Mount Holyoke College.
Towle, Harriet N., Assistant in Zoölogy, Wellesley College.
Waddill, Samuel F., Washington and Jefferson College.
Williams, John L., Graduate Assistant, New York University.
Woodward, Arthur A., Jr., Student, Oberlin College.
Worden, Frederic G., Student, Dartmouth College.

PHYSIOLOGY

Albrink, Wilhelm S., Assistant in Biology, Yale University.
Allen, Paul J., Graduate Assistant in Botany, University of Rochester.
Armstrong, Charles W. J., Demonstrator in Biology, University of Toronto.
Beck, Naomi E., The University of Chicago.
Blair, John H., Graduate Assistant, Wesleyan University.
Bliss, Alfred F., Columbia University.
Briscoe, Priscilla M., Graduate Student, Ohio State University.
Casey, Margaret T., Graduate Assistant in Physiology, Mount Holyoke College.
Crowell, Hamblin H., Graduate Assistant, Ohio State University.
Curtis, Howard J., Fellow, Rockefeller Foundation.
Grave, Caswell, II, Assistant, Washington University.
Henson, Margaret, Assistant in Physiology, Wellesley College.
Levine, Harry Philip, Zoölogy Instructor, University of Vermont.
Martin, Rosemary D. C., Assistant, University of Toronto.
Moore, Imogene, Instructor in Zoölogy, New Jersey College for Women.
Mullins, Lorin J., University of California.
O'Brien, John P., Johns Hopkins University.
Owens, William C., St. John's College.
Smith, Audrey U., Assistant in Physiology, Vassar College.
Von Dach, Herman, Assistant in Zoölogy, Ohio State University.
Wieghard, Charlotte, 4544 Harris Avenue, St. Louis, Missouri.
Wilson, John W., Graduate Assistant in Zoölogy, Duke University.

PROTOZOÖLOGY

Bevel, Nell H., Assistant in Zoölogy, Duke University.
Burbanck, William D., Graduate Assistant, The University of Chicago.
Cole, Roger M., Undergraduate Assistant, Massachusetts State College.
Ewing, William H., Fellow in Biology, Washington and Jefferson College.

Finkelstein, Nathaniel, Johns Hopkins University.
Hierholzer, Carolyn Anne, Instructor in Biology, Adelphia College.
Kornblum, Lucile, Student, Columbia University.
Mayo, Mercedes, Assistant Professor of Biology, Universidad de la Habana.
Wells, Wayne W., Associate Professor of Science, Southern Oregon State Normal.
Wilkinson, Elizabeth J., Student, Columbia University.

INVERTEBRATE ZOÖLOGY

Acosta, Josefina, Goucher College.
Alexander, Robert S., Graduate Assistant, Amherst College.
Arnstein, Margery, Simmons College.
Belda, Walter H., Graduate Student, Johns Hopkins University.
Bigler, Frances B., Western Reserve University.
Brown, Henry, Student, College of the City of New York.
Cooney, Marilyn R., Student, Smith College.
Crane, Todd, Student, Wilson College.
Davis, James O., Graduate Assistant in Zoölogy, University of Missouri.
DeLisa, Dominick A., Student, Union College.
Deringer, Margaret K., Johns Hopkins University.
Dobbelaar, Mark E., Teacher of Science, Oradell High School.
Fahl, Helen, Student, Oberlin College.
Ferguson, Frederick P., Undergraduate Assistant, Wesleyan University.
Fleming, Robert S., Science Critic Teacher, East Carolina Teachers College.
Fraser, Lemuel A., Student, American University.
Gale, Shirley, Radcliffe College.
Graves, E. Irene, Senior Instructor in Biology, Bridgewater State Teachers College.
Griffiths, Raymond B., Graduate Research Assistant, Princeton University.
Haines, William J., Wabash College.
Hall, Lydia R., Graduate Assistant, Mount Holyoke College.
Hamann, Cecil B., Assistant, Purdue University.
Harris, Nellie R., Undergraduate Assistant, Montclair State Teachers College.
Hoagland, Mary, Swarthmore College.
Jaeger, Lucena, Graduate Assistant in Zoölogy, University of Missouri.
Jordon, Elizabeth L., Barnard College.
Joseph, Samuel, Student Laboratory Assistant, DePauw University.
Kellogg, Margaret P., Graduate Student, Cornell University.
Kerrigan, Sylvia, Graduate Assistant, University of Cincinnati.
Linscheid, Alfred G., Western Reserve University.
Love, Genevieve, Pennsylvania College for Women.
McDonald, Brown, Laboratory Assistant, DePauw University.
Morrison, Peter R., Swarthmore College.
Nadler, Evelyn R., Brooklyn College.
Pierson, Mary E., Graduate Assistant, Mount Holyoke College.
Reyer, Randall W., Cornell University.
Rollason, Herbert D., Jr., Middlebury College.
Root, Charlotte C., Student, Mount Holyoke College.
Ryan, Thomas I., Instructor, Boston College.
Sackett, John T., Graduate, University of Pennsylvania.
Sanders, Mary Elizabeth, Depauw University.
Schaeffer, Bobb, Graduate Student, Columbia University.
Schneider, Mathilda E. C., University of Illinois.
Sheehan, Eleanor L., Instructor, University of New Hampshire.
Smith, Ralph I., Harvard University.

Snedecor, James, Student, Iowa State College.
Sperry, Roger W., Oberlin College.
Taber, Elsie, Instructor in Biology, Lander College.
Towle, Harriet N., Assistant in Zoölogy, Wellesley College.
Trowbridge, Carolyn F., University of Iowa.
Ward, Helen L., Assistant in Biology, Purdue University.
Welch, d'Alte A., Johns Hopkins University.
Wells, Lorna A., Graduate Assistant, Oberlin College.
Williams, Edith M., Student, Elmira College.

4. TABULAR VIEW OF ATTENDANCE

	1934	1935	1936	1937	1938
Investigators—Total	323	315	359	391	380
Independent	222	208	226	256	246
Under Instruction	49	56	76	74	53
Research Assistants	52	51	57	61	81
Students—Total	131	130	138	133	132
Zoölogy	54	55	55	57	54
Protozoölogy	11	16	17	16	10
Embryology	30	33	34	35	34
Physiology	23	20	22	16	22
Botany	13	6	10	9	12
Total Attendance	454	445	497	524	512
Less Persons Registered as Both Students and Investigators	15	16	24	13	16
	439	429	473	511	496
Institutions Represented—Total	131	143	158	165	151
By Investigators	98	111	120	134	125
By Students	75	70	77	79	67
Schools and Academies Represented					
By Investigators	1	—	2	3	4
By Students	5	3	3	2	1
Foreign Institutions Represented					
By Investigators	4	7	9	16	14
By Students	1	1	5	—	3

5. SUBSCRIBING AND COÖPERATING INSTITUTIONS IN 1938

American University
Amherst College
Barnard College
Belgian American Education Foundation, Inc.
Bowdoin College
Brothers College of Drew University
Brown University
Bryn Mawr College
Carnegie Institute of Washington
College of Physicians and Surgeons
College of William and Mary
Columbia University
Purdue University
Radcliffe College
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University
St. John's College
Smith College
Spring Hill College
State University of Iowa
Swarthmore College
Syracuse University
Temple University

Connecticut College
Cornell University Medical College
Dalhousie University
Dartmouth College
DePauw University
Duke University
Elmira College
General Education Board
Goucher College
Harvard University
Harvard University Medical School
Hunter College
Industrial & Engineering Chemistry, of the American Chemical Society
Iowa State College
Johns Hopkins University
Kenyon College
Eli Lilly & Company
Long Island University
Massachusetts State College
Memorial Hospital, New York City
Mount Holyoke College
New York State Department of Health
New York University
New York University Medical School
Northwestern University
Oberlin College
Pennsylvania College for Women
Princeton University
Toledo University
Tufts College
Union College
University of Chicago
University of Cincinnati
University of Illinois
University of Maryland Medical School
University of Minnesota
University of Missouri
University of Notre Dame
University of Pennsylvania
University of Pittsburgh
University of Rochester
University of Rochester Medical School
University of Vermont
University of Virginia
Vanderbilt University Medical School
Vassar College
Wabash College
Washington University
Washington University Medical School
Wellesley College
Wesleyan University
Western Reserve University
Wheaton College
Williams College
Wilson College
Yale University

6. EVENING LECTURES, 1938

Tuesday, June 21
Dr. E. H. Myers . . . . " Life Cycle of Foraminifera."

Friday, July 1
Dr. M. H. Jacobs . . . . " Blood and Zoölogical Classification."

Friday, July 8
Dr. S. O. Mast . . . . " The Synthesis of Living Substance, as Exemplified in Chilomonas paramecium."

Friday, July 15
Dr. G. H. Parker . . . . " The Color Changes in Animals and Neurohumoral Transmission."

Wednesday, July 20
Dr. Robert Chambers and
Dr. William Duryee . . . . " Micromanipulation Studies on Cells and Nuclei."

Friday, July 22
Dr. O. E. Schotté . . . . " Induction of Embryonic Organs in Regenerates and Neoplasms."

Friday, July 29
Dr. Eduard Uhlenhuth . . . . " A Quantitative Approach to the Secretion Process of the Thyroid."

Friday, August 5
Dr. Robert Chambers .... “Structural Aspects of Cell Division.”

Tuesday, August 9
Dr. E. G. Conklin and
Dr. F. R. Lillie .... “Informal Memorial of the Fiftieth Anniversary of the Founding of the Marine Biological Laboratory.”

Friday, August 12
Dr. L. G. Barth .... “Studies of the Factors Influencing Regeneration.”

Friday, August 19
Mr. Columbus Iselin .... “The Influence of Fluctuations in the Major Ocean Currents on the Climate and the Fisheries.”

Friday, August 26
Dr. Peter Gray .... “The Possibility of Affecting Developmental Patterns by Electrical Means.”

Thursday, September 1 (Under the joint auspices of the Genetics Society of America and the Marine Biological Laboratory)
Dr. H. J. Muller .... “The Remaking of Chromosomes.”

7. SHORTER SCIENTIFIC PAPERS, 1938

Tuesday, July 5
Dr. W. H. Newton .... “Endocrine Activity of the Placenta in Mice.”
Dr. J. K. W. Ferguson and
Dr. H. O. Haterius .... “Evidence for Hormonal Control of Uterine Motility by the Hypophysis in the Rabbit.”
Dr. Roberts Rugh .... “Experimental Studies on the Genital System of the Male Anuran.”

Tuesday, July 12
Dr. G. W. Kidder and
Dr. C. A. Stuart .... “The Rôle of Chromogenic Bacteria in Ciliate Growth.”
Mr. J. A. Smith .... “Some Effects of Temperature on the Reproduction of Chilomonas paramecium.”
Dr. D. L. Hopkins .... “Adjustment of the marine Amoeba, Flabellula mira Schaeffer, to changes in the Total Salt Concentration of the Outside Medium.”
Mr. C. L. Claff .... “Phenomena of Excystment in Colpoda cucullus.”

Tuesday, July 19
Dr. K. C. Fisher and
Mr. R. Öhnell .... “The Steady State Frequency of the Embryonic Fish Heart at Different Cyanide Concentrations.”

Dr. Lena A. Lewis	"Studies on the Refractory State Resulting from the Repeated Injections of Adrenal Extract."
Dr. Emil Bozler	"Action Potentials of Visceral Smooth Muscles."
Dr. L. Irving	"Rhythmical Changes in Blood Flow Through Muscles."
Tuesday, July 26	
Dr. B. H. Willier and Dr. Mary E. Rawles	"Skin Transplants between Embryos of Different Breeds of Fowl."
Dr. Arthur Colwin	"Induction by Cauterization in the Amphibian Egg."
Dr. Viktor Hamburger	"The Innervation of Transplanted Limbs in Chick Embryos."
Dr. Paul Weiss	"The Effect of Mechanical Stress on Cartilage Differentiated in Vitro."
Tuesday, August 2	
Dr. J. P. Visscher	"Some Recent Studies on Barnacles."
Dr. E. R. Jones, Jr.	"Observations on some of the Lower Turbellaria of the Eastern United States."
Dr. d'A. A. Welch	"Some Problems of Distribution and Variation in the Hawaiian Tree Snail Achatinella."
Tuesday, August 16	
Dr. D. P. Costello	"Studies on Fragments of Centrifuged Nereis Eggs."
Dr. Victor Schechter	"Calcium and Magnesium in Relation to the Longevity of Egg Cells."
Dr. J. B. Buck and Dr. R. D. Boche	"Some Properties of Living Chromosomes."
Dr. A. M. Lucas	"Some Cytological Studies on Virus-Infected Cells."
Dr. W. R. Duryee	"A Microdissection Study of Amphibian Chromosomes."
Tuesday, August 23	
Mr. Karl Wilbur	"The Relation of the Magnesium Ion to Ultraviolet Stimulation in the Nereis Egg."
Dr. E. Eleanor Carothers	"Cytological Effects of X-Rays on Grasshopper Embryos."
Dr. J. Furth	"Quantitative Studies on the Effect of X-Rays on Mammalian Cells, and on the Mode of X-Ray Action."
Dr. P. S. Henshaw	"The Effect of X-Rays on Arbacia punctulata Sperm."
Dr. T. N. White	"Recovery of Arbacia Eggs from High Intensity X-Ray Effects."

8. GENERAL SCIENTIFIC MEETING, 1938

Tuesday, August 30

Miss A. M. Melland	"Isolation of Salivary Gland Nuclei."
Mr. Glenn H. Algire	"Cytological Studies on the Living Thyroid of the Salamander."
Dr. Ralph H. Cheney	"Micro-Structural Changes in Muscle Fibers after Caffeine."
Dr. Carl C. Speidel	"Some Features of Contraction Nodes and Retraction Clots as Observed in Single Fibers of Cardiac and Skeletal Muscle of Both Vertebrates and Invertebrates."
Dr. Michael J. D. White	"The Heteropycnosis of Sex Chromosomes and its Interpretation in Terms of Spiral Structure."
Dr. John P. Turner	"Mitochondria and other Inclusions in the Ciliate Tillina canalifera."
Dr. Robert Chambers	"Cytoplasmic Inclusions and Matrix of the Arbacia Egg."
Dr. M. J. Kopac	"The Devaux Effect at Oil-Protoplasm Interfaces."
Dr. M. H. Jacobs and Dr. A. K. Parpart	"Further Studies on the Permeability of the Erythrocyte to Ammonium Salts."
Mr. A. J. Dziemian and Dr. A. K. Parpart	"Permeability and the Lipoid Content of the Erythrocyte."
Mr. Louis-Paul Dugal and Dr. Laurence Irving	"The Relation of the Shell to Anaerobic Metabolism in Venus mercenaria."
Dr. Alexander Sandow and Dr. Kenneth Moritz	"Tension Output of Muscles in Hypotonic Solutions."
Dr. Dwight L. Hopkins	"The Mechanism for the Control of the Intake and the Output of Water by the Vacuoles in the Marine Amoeba, Flabellula mira Schaeffer."
Dr. N. S. R. Maloeuf	"On the Kidney of the Crayfish and the Uptake of Chlorid from Fresh Water by this Animal."
Dr. N. S. R. Maloeuf	"The Osmo-regulative Function of the Alimentary Tract of the Earthworm, and on the Uptake of Chlorid from Fresh Water by this Animal."

Dr. Ethel Browne Harvey	"Development of Half-Eggs of Chaetopterus Obtained by Centrifugal Force."
Dr. Paul S. Henshaw	"The Question of Whether the Delay in Cleavage of Arbacia Eggs Produced with X-Rays is Caused by a General Slowing of the Cleavage Process or by a Block at Some Particular Stage."
Mr. E. L. Chambers and Dr. Robert Chambers	"The Resistance of Fertilized Arbacia Eggs to Immersion in KCl and NaCl Solutions."
Dr. Albert E. Navez	"Indolphenoloxidase in Arbacia Eggs and the Nadi Reaction."
Dr. K. C. Cole and Dr. Howard J. Curtis	"Electric Impedance of Nerve During Activity."
Dr. Frank A. Geldard	"The Vibratory Response of the Skin and its Relation to Pressure Sensitivity."
Dr. E. Alfred Wolf	"Reversal of Phototropic Reaction in Daphnia by the Use of Photosensitizing Dyes."
Dr. Carl C. Speidel	"Motion Picture Showing Microscopic Changes in Fibers of Cardiac and Skeletal Muscle of Invertebrates and Vertebrates during Contraction, Retraction, and Clotting."
Dr. W. R. Duryee	"The Action of Direct Currents on the Cell Nucleus."
Dr. W. R. Duryee	"Hydration and Dehydration of Follicle Cell Nuclei."
Wednesday, August 31	
Dr. Herbert Elftman	"The Function of Muscles in Locomotion."
Dr. William J. Bowen	"The Effects of Copper and of Vanadium on the Frequency of Division."
Dr. Sarah Bedichek	"Sex Balance in the Progeny of Triploid Drosophila."
Dr. Eduard Uhlenhuth, Mr. James U. Thompson and Mr. Joseph E. Schenthal	"The Antihormone Problems in the Salamander."
Dr. Roberts Rugh	"The Effect of the Sex-Stimulating Factor of the Anterior Pituitary Gland on the Testis of the Bullfrog."

Dr. J. Paul Visscher	"Studies on Barnacle Larvae."
Dr. Grace Townsend	"The Spawning Reaction of Nereis limbata with Emphasis Upon Chemical Stimulation."
Dr. Grace Townsend	"Physiological Assays Concerning the Nature of Fertilizin."
Dr. Elbert C. Cole	"A Study of the Integument of the Squid, During Staining with Methylene Blue."
Mr. Carl C. Smith and Mr. Louis Levin	"The Use of the Clam Heart as a Test Object for Acetylcholine."
Dr. Oscar W. Richards and Miss Katharine J. Hawley	"The Elimination of Molds."
Dr. S. E. Pond, Mr. E. P. Little, Mr. A. M. Smith, and Mr. J. D. Graham	"A Comparative Study of Water Aspirators."

PAPERS READ BY TITLE

Dr. C. A. Angerer	"The Effect of Electric Current on the Physical Consistency of Sea Urchin Eggs."
Mr. C. W. J. Armstrong and Dr. K. C. Fisher	"The Effect of Sodium Azide on the Frequency of the Embryonic Fundulus Heart."
Mr. Robert Ballantine	"Reducing Activity of Fertilized and Unfertilized Arbacia Eggs."
Dr. Ludwig von Bertalanffy	"Studies on the Mechanism of Growth in Planaria maculata."
Mrs. Ruth Snyder Cooper	"Probable Absence of a Chromatophore Activator in Limulus polyphemus."
Mr. C. G. Grand	"Intracellular pH Studies on the Ova of Mactra solidissima."
Dr. W. R. Duryee	"The Action of Fixatives on the Isolated Cell Nucleus."
Dr. Adolph Elwyn	"The Melanophore-Expanding Activity of the Ascidian Neural Gland."
Mr. Richard W. Foster, Mr. John D. Crawford and Dr. Albert E. Navez	"Cardiac Rhythm in Pecten irradians (Lamarck)."
Dr. Stephen Karády	"The Alarm Reaction and Adaptation Syndrome in Lower Vertebrates (Fundulus majalis)."

Dr. M. J. Kopac	"Micro-estimation of Protein Adsorption at Oil-Protoplasm Interfaces."
Dr. M. J. Kopac and Dr. R. Chambers	"Effect of the Vitelline Membrane on Coalescence of Arbacia Eggs with Oil-drops."
Dr. George Saslow	"The Osmotic Pressure of Gum Acacia Solutions."
Dr. A. A. Schaeffer	"Differences Between Scottish and American Amebas of the Species Chaos diffluens Müller."
Dr. Victor Schechter	"Induction in Griffithsia."
Dr. Victor Schechter	"Bacteria in Relation to Longevity of Egg Cells."
Dr. J. N. Stannard	"The Effect of Sodium Azide on the Respiration of Frog Muscle."
Dr. A. J. Waterman	"Respiratory Stimulants and Gastrulation in Arbacia."
Dr. Ralph Wichterman	"Does Transfer of Pronuclei ever Occur in Conjugation of Paramecium caudatum?"
Dr. E. Alfred Wolf and Mr. A. S. Levenson	"Studies in Calcification. IV. A Contribution to the Problem of Skeletal Calcification in the Teleost, Fundulus heteroclitus."
Dr. Opal M. Wolf	"Mitotic Activity of the Islands of Langerhans and Parathyroids of Rats Following Pituitary Extract and Colchicine Injections."
Dr. Opal M. Wolf	"Oviducts of Pituitary Stimulated Females, Rana pipiens."
Miss R. A. Young	"The Effects of Roentgen Irradiation on Cleavage and Early Development in the Annelid, Chaetopterus pergamentaceus."
Mr. E. Zwilling	"The Effect of Perisarc Removal on Regeneration in Tubularia crocea."

DEMONSTRATIONS

Wednesday, August 31

Dr. Michael J. D. White	"The Spiral Structure of Animal Chromosomes."
Dr. P. S. Henshaw	"Cellular Abnormalities Produced by X-Rays."
Dr. K. S. Cole and H. J. Curtis	"Electrical Impedance Changes in the Squid Giant Axon Following Excitation."

Dr. E. R. Clark and Mrs. Eleanor Linton Clark	a) “Marked Macrophages.” b) “Arterio-venous Anastomoses as Observed in the Living Mammal.”
Mr. C. H. Norris	“Method of Studying Elastic Tension of Marine Eggs.”
Mr. G. H. Algire	“Apparatus for the Cytological Study of the Thyroid in the Living Salamander.”
Dr. J. P. Turner	“Mitochondria and Other Inclusions in the Ciliate Tillina canalifera.”
Mr. A. S. Levenson	“Microscopic Sections through Head and Trunk Regions of Fundulus heteroclitus, Prepared by the Gömöre Silver Nitrate Method for the Study of Calcification.”
Dr. A. K. Parpart and Mr. S. B. Young	“A Simple Glass Electrode System.”
Dr. S. E. Pond and Mr. E. P. Little	“Water Aspirator Tests and Comparisons.”
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Evans, Mrs. Glendower, 12 Otis Place, Boston, Massachusetts.
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Gardiner, Mrs. E. G., Woods Hole, Massachusetts.
Jackson, Miss M. C., 88 Marlboro St., Boston, Massachusetts.
Jackson, Mr. Chas. C., 24 Congress St., Boston, Massachusetts.
King, Mr. Chas. A.
Lee, Mrs. Frederic S., 279 Madison Ave., New York City.
Lee, Prof. F. S., College of Physicians and Surgeons, New York City.
Lewis, Prof. W. H., Johns Hopkins University, Baltimore, Maryland.
Lowell, Mr. A. L., 17 Quincy St., Cambridge, Mass.
McMurrich, Prof. J. P., Toronto, Canada.
Means, Dr. J. H., 15 Chestnut St., Boston, Mass.
Moore, Dr. George T., Missouri Botanical Gardens, St. Louis, Mo.
Morgan, Mr. J. Pierpont, Jr., Wall and Broad Sts., New York City.
Morgan, Prof. T. H., Director of Biological Laboratory, California Institute of Technology, Pasadena, California.
Morgan, Mrs. T. H., Pasadena, California.
Morrill, Dr. A. D., Hamilton College, Clinton, N. Y.
Noyes, Miss Eva J.
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania.
Sears, Dr. Henry F., 86 Beacon St., Boston, Massachusetts.
Shedd, Mr. E. A.
Thorndike, Dr. Edward L., Teachers College, Columbia University, New York City.
Treadwell, Prof. A. L., Vassar College, Poughkeepsie, N. Y.
Trelease, Prof. William, University of Illinois, Urbana, Illinois.
Wallace, Louise B., 359 Lytton Avenue, Palo Alto, Calif.
Wilson, Dr. E. B., Columbia University, New York City.

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Abramowitz, Dr. Alexander A., Biological Laboratories, Harvard University, Cambridge, Massachusetts.

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Addison, Dr. W. H. F., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.

Adolph, Dr. Edward F., University of Rochester Medical School, Rochester, New York.

Allee, Dr. W. C., The University of Chicago, Chicago, Illinois.

Allyn, Dr. Harriet M., Mount Holyoke College, South Hadley, Massachusetts.

Amberson, Dr. William R., Department of Physiology, University of Maryland, School of Medicine, Lombard and Greene Streets, Baltimore, Maryland.

Anderson, Dr. E. G., California Institute of Technology, Pasadena, California.

Anderson, Dr. Rubert S., Guyot Hall, Princeton University, Princeton, New Jersey.

Armstrong, Dr. Philip B., Syracuse University, Syracuse, New York.

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Ball, Dr. Eric G., Johns Hopkins Medical School, Baltimore, Maryland.

Ballard, Dr. William W., Dartmouth College, Hanover, New Hampshire.

Bard, Prof. Philip, Johns Hopkins Medical School, Baltimore, Maryland.

Barron, Dr. E. S. Guzman, Department of Medicine, The University of Chicago, Chicago, Illinois.

Barth, Dr. L. G., Department of Zoölogy, Columbia University, New York City.

Beadle, Dr. G. W., School of Biological Sciences, Stanford University, California.

Beckwith, Dr. Cora J., Vassar College, Poughkeepsie, New York.

Behre, Dr. Elinor H., Louisiana State University, Baton Rouge, Louisiana.

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Bigelow, Dr. H. B., Museum of Comparative Zoölogy, Cambridge, Massachusetts.

Bigelow, Prof. R. P., Massachusetts Institute of Technology, Cambridge, Massachusetts.

Binford, Prof. Raymond, Guilford College, Guilford College, North Carolina.

Bissonnette, Dr. T. Hume, Trinity College, Hartford, Connecticut.

Blanchard, Prof. Kenneth C., Washington Square College, New York University, New York City.

Bodine, Dr. J. H., Department of Zoölogy, State University of Iowa, Iowa City, Iowa.

Boring, Dr. Alice M., Yenching University, Peking, China.

Bozler, Dr. Emil, Ohio State University, Columbus, Ohio.

Bradley, Prof. Harold C., University of Wisconsin, Madison, Wisconsin.

Bridges, Dr. Calvin B., California Institute of Technology, Pasadena, California.

Bronfenbrenner, Dr. Jacques J., Department of Bacteriology, Washington University Medical School, St. Louis, Missouri.

Bronk, Dr. D. W., University of Pennsylvania, Philadelphia, Pennsylvania.

Brooks, Dr. S. C., University of California, Berkeley, California.

Brown, Dr. Dugald E. S., New York University, College of Medicine, New York City.

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Bumpus, Prof. H. C., Duxbury, Massachusetts.

Byrnes, Dr. Esther E., 1803 North Camac Street, Philadelphia, Pennsylvania.

Calkins, Prof. Gary N., Columbia University, New York City.

Calvert, Prof. Philip P., University of Pennsylvania, Philadelphia, Pennsylvania.

Cannan, Prof. R. K., New York University College of Medicine, 477 First Avenue, New York City.

Carlson, Prof. A. J., Department of Physiology, The University of Chicago, Chicago, Illinois.

Carothers, Dr. E. Eleanor, Department of Zoölogy, State University of Iowa, Iowa City.

Carpenter, Dr. Russell L., Tufts College, Tufts College, Massachusetts.

Carroll, Prof. Mitchell, Franklin and Marshall College, Lancaster, Pennsylvania.

Carver, Prof. Gail L., Mercer University, Macon, Georgia.

Cattell, Dr. McKeen, Cornell University Medical College, 1300 York Avenue, New York City.

Cattell, Prof. J. McKeen, Garrison-on-Hudson, New York.
Cattell, Mr. Ware, Garrison-on-Hudson, New York.
Chambers, Dr. Robert, Washington Square College, New York University, Washington Square, New York City.
Cheney, Dr. Ralph H., Biology Department, Long Island University, Brooklyn, New York.
Chidester, Prof. F. E., Auburndale, Massachusetts.
Child, Prof. C. M., Jordan Hall, Stanford University, California.
Claff, Mr. C. Lloyd, Department of Biology, Brown University, Providence, Rhode Island.
Clark, Prof. E. R., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.
Clark, Dr. Leonard B., Union College, Schenectady, New York.
Cleland, Prof. Ralph E., Indiana University, Bloomington, Indiana.
Clowes, Dr. G. H. A., Eli Lilly and Company, Indianapolis, Indiana.
Coe, Prof. W. R., Yale University, New Haven, Connecticut.
Cohn, Dr. Edwin J., 183 Brattle Street, Cambridge, Massachusetts.
Cole, Dr. Elbert C., Department of Biology, Williams College, Williamstown, Massachusetts.
Cole, Dr. Kenneth S., College of Physicians and Surgeons, Columbia University, 630 W. 168th Street, New York City.
Cole, Dr. Leon J., College of Agriculture, Madison, Wisconsin.
Collett, Dr. Mary E., Western Reserve University, Cleveland, Ohio.
Colton, Prof. H. S., Box 601, Flagstaff, Arizona.
Coonfield, Dr. B. R., Brooklyn College, 80 Willoughby Street, Brooklyn, New York.
Copeland, Prof. Manton, Bowdoin College, Brunswick, Maine.
Costello, Dr. Donald P., Department of Zoölogy, University of North Carolina, Chapel Hill, North Carolina.
Costello, Dr. Helen Miller, Department of Zoölogy, University of North Carolina, Chapel Hill, North Carolina.
Cowdry, Dr. E. V., Washington University, St. Louis, Missouri.
Crampton, Prof. H. E., Barnard College, Columbia University, New York City.
Crane, Mrs. C. R., Woods Hole, Massachusetts.
Crowell, Dr. P. S., Jr., Department of Zoölogy, Miami University, Oxford, Ohio.
Curtis, Dr. Maynie R., Crocker Laboratory, Columbia University, New York City.
Curtis, Prof. W. C., University of Missouri, Columbia, Missouri.
Dan, Dr. Katsuma, Misaki Biological Station, Misaki, Japan.

Davis, Dr. Donald W., College of William and Mary, Williamsburg, Virginia.

Dawson, Dr. A. B., Harvard University, Cambridge, Massachusetts.

Dawson, Dr. J. A., The College of the City of New York, New York City.

Dederer, Dr. Pauline H., Connecticut College, New London, Connecticut.

Diller, Dr. William F., Dartmouth College, Hanover, New Hampshire.

Dodds, Prof. G. S., Medical School, University of West Virginia, Morgantown, West Virginia.

Dolley, Prof. William L., University of Buffalo, Buffalo, New York.

Donaldson, Dr. John C., University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania.

DuBois, Dr. Eugene F., Cornell University Medical College, 1300 York Avenue, New York City.

Duggar, Dr. Benjamin M., University of Wisconsin, Madison, Wisconsin.

Dungay, Dr. Neil S., Carleton College, Northfield, Minnesota.

Duryee, Dr. William R., Department of Biology, Washington Square College, New York University, New York City.

Edwards, Dr. D. J., Cornell University Medical College, 1300 York Avenue, New York City.

Ellis, Dr. F. W., Monson, Massachusetts.

Fauré-Fremiet, Prof. Emmanuel, Collège de France, Paris, France.

Ferguson, Dr. James K. W., Department of Physiology, Ohio State University, Columbus, Ohio.

Fischer, Dr. Ernst, Department of Physiology, Medical College of Virginia, Richmond, Virginia.

Fisher, Dr. Kenneth C., Department of Biology, University of Toronto, Toronto, Canada.

Fleisher, Dr. Moyer S., School of Medicine, St. Louis University, St. Louis, Missouri.

Forbes, Dr. Alexander, Harvard University Medical School, Boston, Massachusetts.

Fry, Dr. Henry J., Cornell University Medical College, 1300 York Avenue, New York City.

Furth, Dr. Jacob, Cornell University Medical College, 1300 York Avenue, New York City.

Gage, Prof. S. H., Cornell University, Ithaca, New York.

Galtsoff, Dr. Paul S., 420 Cumberland Avenue, Somerset, Chevy Chase, Maryland.

Garrey, Prof. W. E., Vanderbilt University Medical School, Nashville, Tennessee.

Gates, Prof. R. Ruggles, University of London, London, England.

Geiser, Dr. S. W., Southern Methodist University, Dallas, Texas.

Gerard, Prof. R. W., The University of Chicago, Chicago, Illinois.

Glaser, Prof. O. C., Amherst College, Amherst, Massachusetts.

Goldforb, Prof. A. J., College of the City of New York, Convent Avenue and 139th Street, New York City.

Goodrich, Prof. H. B., Wesleyan University, Middletown, Connecticut.

Gottschall, Dr. Gertrude Y., 230 Central Park West, New York City.

Graham, Dr. J. Y., University of Alabama, University, Alabama.

Grave, Prof. B. H., DePauw University, Greencastle, Indiana.

Grave, Prof. Caswell, Washington University, St. Louis, Missouri.

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Gregory, Dr. Louise H., Barnard College, Columbia University, New York City.

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Hamburger, Dr. Viktor, Department of Zoölogy, Washington University, St. Louis, Missouri.

Hance, Dr. Robert T., University of Pittsburgh, Pittsburgh, Pennsylvania.

Hargitt, Prof. George T., Department of Zoölogy, Duke University, Durham, North Carolina.

Harman, Dr. Mary T., Kansas State Agricultural College, Manhattan, Kansas.

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Hartline, Dr. H. Keffer, University of Pennsylvania, Philadelphia, Pennsylvania.

Harvey, Dr. Ethel Browne, 48 Cleveland Lane, Princeton, New Jersey.

Harvey, Dr. E. Newton, Guyot Hall, Princeton University, Princeton, New Jersey.

Hayden, Dr. Margaret A., Wellesley College, Wellesley, Massachusetts.

Hayes, Dr. Frederick R., Zoölogical Laboratory, Dalhousie University, Halifax, Nova Scotia.

Haywood, Dr. Charlotte, Mount Holyoke College, South Hadley, Massachusetts.

Hazen, Dr. T. E., Barnard College, Columbia University, New York City.

Hecht, Dr. Selig, Columbia University, New York City.

Heilbrunn, Dr. L. V., Department of Zoölogy, University of Pennsylvania, Philadelphia, Pennsylvania.

Hendee, Dr. Esther Crissey, Russell Sage College, Troy, New York.

Henshaw, Dr. Paul S., Memorial Hospital, 2 West 106th Street, New York City.

Hess, Prof. Walter N., Hamilton College, Clinton, New York.

Hibbard, Dr. Hope, Department of Zoölogy, Oberlin College, Oberlin, Ohio.

Hill, Dr. Samuel E., Department of Biology, Princeton University, Princeton, New Jersey.

Hisaw, Dr. F. L., Harvard University, Cambridge, Massachusetts.

Hoadley, Dr. Leigh, Harvard University, Cambridge, Massachusetts.

Höber, Dr. Rudolf, University of Pennsylvania, Philadelphia, Pennsylvania.

Hodge, Dr. Charles, IV., Temple University, Department of Zoölogy, Philadelphia, Pennsylvania.

Hogue, Dr. Mary J., 503 N. High Street, West Chester, Pennsylvania.

Hollaender, Dr. Alexander, c/o National Institute of Health, Laboratory of Ind. Hygiene, 25th and E Street, N.W., Washington, D. C.

Hooker, Prof. Davenport, University of Pittsburgh, School of Medicine, Department of Anatomy, Pittsburgh, Pennsylvania.

Hopkins, Dr. Dwight L., Mundelein College, 6363 Sheridan Road, Chicago, Illinois.

Hopkins, Dr. Hoyt S., New York University, College of Dentistry, New York City.

Howe, Dr. H. E., 2702 36th Street, N.W., Washington, D. C.

Howland, Dr. Ruth B., Washington Square College, New York University, Washington Square East, New York City.

Hoyt, Dr. William D., Washington and Lee University, Lexington, Virginia.

Hyman, Dr. Libbie H., 85 West 166th Street, New York City.

Irving, Prof. Laurence, Swarthmore College, Swarthmore, Pennsylvania.

Jackson, Prof. C. M., University of Minnesota, Minneapolis, Minnesota.

Jacobs, Prof. Merkel H., School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.
Jenkins, Dr. George B., George Washington University, 1335 M Street, N.W., Washington, D. C.
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Jewett, Prof. J. R., 44 Francis Avenue, Cambridge, Massachusetts.
Johlin, Dr. J. M., Vanderbilt University Medical School, Nashville, Tennessee.
Jones, Dr. E. Ruffin, Jr., College of William and Mary, Norfolk, Virginia.
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Kille, Dr. Frank R., Swarthmore College, Swarthmore, Pennsylvania.
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King, Dr. Helen D., Wistar Institute of Anatomy and Biology, 36th Street and Woodland Avenue, Philadelphia, Pennsylvania.
King, Dr. Robert L., State University of Iowa, Iowa City, Iowa.
Kingsbury, Prof. B. F., Cornell University, Ithaca, New York.
Knower, Prof. H. McE., Woods Hole, Massachusetts.
Knowlton, Prof. F. P., Syracuse University, Syracuse, New York.
Kopac, Dr. M. J., Washington Square College, New York University, New York City.
Korr, Dr. I. M., Department of Physiology, Washington Square College, New York University, New York City.
Krahl, Dr. M. E., Lilly Research Laboratories, Indianapolis, Indiana.
Krieg, Dr. Wendell J. S., New York University, College of Medicine, 477 First Avenue, New York City.
Lancefield, Dr. D. E., Queens College, Flushing, New York.
Lange, Dr. Mathilde M., Wheaton College, Norton, Massachusetts.
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Lillie, Prof. Frank R., The University of Chicago, Chicago, Illinois.
Lillie, Prof. Ralph S., The University of Chicago, Chicago, Illinois.
Linton, Prof. Edwin, University of Pennsylvania, Philadelphia, Pennsylvania.
Loeb, Prof. Leo, Washington University Medical School, St. Louis, Missouri.

Lowther, Mrs. Florence deL., Barnard College, Columbia University, New York City.

Lucas, Dr. Alfred M., Zoölogical Laboratory, Iowa State College, Ames, Iowa.

Lucas, Dr. Miriam Scott, Department of Zoölogy, Iowa State College, Ames, Iowa.

Lucké, Prof. Balduin, University of Pennsylvania, Philadelphia, Pennsylvania.

Luscombe, Mr. W. O., Woods Hole, Massachusetts.

Lynch, Dr. Clara J., Rockefeller Institute, 66th Street and York Avenue, New York City.

Lynch, Dr. Ruth Stocking, Maryland State Teachers College, Towson, Maryland.

MacCardle, Dr. Ross C., School of Medicine, Duke University, Durham, North Carolina.

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McClung, Prof. C. E., University of Pennsylvania, Philadelphia, Pennsylvania.

McGregor, Dr. J. H., Columbia University, New York City.

Macklin, Dr. Charles C., School of Medicine, University of Western Ontario, London, Canada.

Magruder, Dr. Samuel R., Department of Anatomy, Tufts Medical School, Boston, Massachusetts.

Malone, Prof. E. F., College of Medicine, University of Cincinnati, Department of Anatomy, Cincinnati, Ohio.

Manwell, Dr. Reginald D., Syracuse University, Syracuse, New York.

Marsland, Dr. Douglas A., Washington Square College, New York University, New York City.

Martin, Prof. E. A., Department of Biology, Brooklyn College, 80 Willoughby Street, Brooklyn, New York.

Mast, Prof. S. O., Johns Hopkins University, Baltimore, Maryland.

Mathews, Prof. A. P., University of Cincinnati, Cincinnati, Ohio.

Matthews, Dr. Samuel A., Thompson Biological Laboratory, Williams College, Williamstown, Massachusetts.

Mavor, Prof. James W., Union College, Schenectady, New York.

Mazia, Dr. Daniel, Department of Zoölogy, University of Missouri, Columbia, Missouri.

Medes, Dr. Grace, Lankenau Research Institute, Philadelphia, Pennsylvania.

Meigs, Dr. E. B., Dairy Division Experimental Station, Beltsville, Maryland.

Meigs, Mrs. E. B., 1736 M Street, N.W., Washington, D. C.

Metcalf, Prof. M. M., 51 Annawan Road, Waban, Massachusetts.

Metz, Prof. Charles W., Johns Hopkins University, Baltimore, Maryland.

Michaelis, Dr. Leonor, Rockefeller Institute, 66th Street and York Avenue, New York City.

Miller, Dr. J. A., Department of Anatomy, University of Michigan, Ann Arbor, Michigan.

Mitchell, Dr. Philip H., Brown University, Providence, Rhode Island.

Moore, Dr. Carl R., The University of Chicago, Chicago, Illinois.

Moore, Prof. J. Percy, University of Pennsylvania, Philadelphia, Pennsylvania.

Morgulis, Dr. Sergius, University of Nebraska, Omaha, Nebraska.

Morrill, Prof. C. V., Cornell University Medical College, 1300 York Avenue, New York City.

Navez, Dr. Albert E., Department of Biology, Milton Academy, Milton, Massachusetts.

Neal, Prof. H. V., Tufts College, Tufts College, Massachusetts.

Nelsen, Dr. Olin E., Department of Zoölogy, University of Pennsylvania, Philadelphia, Pennsylvania.

Newman, Prof. H. H., The University of Chicago, Chicago, Illinois.

Nichols, Dr. M. Louise, Rosemont, Pennsylvania.

Noble, Dr. Gladwyn K., American Museum of Natural History, New York City.

Nonidez, Dr. José F., Cornell University Medical College, 1300 York Avenue, New York City.

Northrop, Dr. John H., The Rockefeller Institute, Princeton, New Jersey.

Okkelberg, Dr. Peter, Department of Zoölogy, University of Michigan, Ann Arbor, Michigan.

Osburn, Prof. R. C., Ohio State University, Columbia, Ohio.

Osterhout, Mrs. W. J. V., Rockefeller Institute, 66th Street and York Avenue, New York City.

Osterhout, Prof. W. J. V., Rockefeller Institute, 66th Street and York Avenue, New York City.

Packard, Dr. Charles, Columbia University, Institute of Cancer Research, 168th Street and Broadway, New York City.

Page, Dr. Irvine H., Lilly Laboratory Clinical Research, Indianapolis City Hospital, Indianapolis, Indiana.

Pappenheimer, Dr. A. M., Columbia University, New York City.

Parker, Prof. G. H., Harvard University, Cambridge, Massachusetts.

Parmenter, Dr. C. L., Department of Zoölogy, University of Pennsylvania, Philadelphia, Pennsylvania.

Parpart, Dr. Arthur K., Princeton University, Princeton, New Jersey.

Patten, Dr. Bradley M., University of Michigan Medical School, Ann Arbor, Michigan.

Payne, Prof. F., University of Indiana, Bloomington, Indiana.

Pearl, Prof. Raymond, Institute for Biological Research, 1901 East Madison Street, Baltimore, Maryland.

Peebles, Prof. Florence, Chapman College, Los Angeles, California.

Pinney, Dr. Mary E., Milwaukee-Downer College, Milwaukee, Wisconsin.

Plough, Prof. Harold H., Amherst College, Amherst, Massachusetts.

Pollister, Dr. A. W., Columbia University, New York City.

Pond, Dr. Samuel E., Marine Biological Laboratory, Woods Hole, Massachusetts.

Pratt, Dr. Frederick H., Boston University, School of Medicine, Boston, Massachusetts.

Prosser, Dr. C. Ladd, Clark University, Worcester, Massachusetts.

Raffel, Dr. Daniel, Institute of Genetics, Academy of Sciences, Moscow, U. S. S. R.

Rand, Dr. Herbert W., Harvard University, Cambridge, Massachusetts.

Rankin, Dr. John S., Biology Department, Amherst College, Amherst, Massachusetts.

Redfield, Dr. Alfred C., Harvard University, Cambridge, Massachusetts.

Reese, Prof. Albert M., West Virginia University, Morgantown, West Virginia.

deRenyi, Dr. George S., Department of Anatomy, University of Pennsylvania, Philadelphia, Pennsylvania.

Reznikoff, Dr. Paul, Cornell University Medical College, 1300 York Avenue, New York City.

Rice, Prof. Edward L., Ohio Wesleyan University, Delaware, Ohio.

Richards, Prof. A., University of Oklahoma, Norman, Oklahoma.

Richards, Dr. O. W., Research Department, Spencer Lens Company, 19 Doat Street, Buffalo, New York.

Riggs, Lawrason, Jr., 120 Broadway, New York City.

Rogers, Prof. Charles G., Oberlin College, Oberlin, Ohio.

Romer, Dr. Alfred S., Harvard University, Cambridge, Massachusetts.

Root, Dr. R. W., Department of Biology, College of the City of New York, Convent Avenue and 139th Street, New York City.

Root, Dr. W. S., College of Physicians and Surgeons, Department of Physiology, 630 West 168th Street, New York City.

Rugh, Dr. Roberts, Department of Zoölogy, Hunter College, New York City.

Saslow, Dr. George, Harvard School of Public Health, 55 Shattuck Street, Boston, Massachusetts.

Sayles, Dr. Leonard P., Department of Biology, College of the City of New York, 139th Street and Convent Avenue, New York City.

Schaeffer, Dr. Asa A., Biology Department, Temple University, Philadelphia, Pennsylvania.

Schechter, Dr. Victor, College of the City of New York, 139th Street and Convent Avenue, New York City.

Schmidt, Dr. L. H., Christ Hospital, Cincinnati, Ohio.

Schrader, Dr. Franz, Department of Zoölogy, Columbia University, New York City.

Schrader, Dr. Sally Hughes, Department of Zoölogy, Columbia University, New York City.

Schotté, Dr. Oscar E., Department of Biology, Amherst College, Amherst, Massachusetts.

Schramm, Prof. J. R., University of Pennsylvania, Philadelphia, Pennsylvania.

Scott, Dr. Allan C., Union College, Schenectady, New York.

Scott, Dr. Ernest L., Columbia University, New York City.

Scott, Prof. William B., 7 Cleveland Lane, Princeton, New Jersey.

Semple, Mrs. R. Bowling, 140 Columbia Heights, Brooklyn, New York.

Severinghaus, Dr. Aura E., Department of Anatomy, College of Physicians and Surgeons, 630 W. 168th Street, New York City.

Shapiro, Dr. Herbert, Department of Biology, Clark University, Worcester, Massachusetts.

Shull, Prof. A. Franklin, University of Michigan, Ann Arbor, Michigan.

Shumway, Dr. Waldo, University of Illinois, Urbana, Illinois.

Sichel, Dr. Ferdinand J. M., University of Vermont, Burlington, Vermont.

Sivickis, Dr. P. B., Pasto Deze 130, Kaunas, Lithuania.

Slifer, Dr. Eleanor H., Department of Zoölogy, State University of Iowa, Iowa City, Iowa.

SMITH, DR. DIETRICH CONRAD, Department of Physiology, University of Maryland, School of Medicine, Lombard and Greene Streets, Baltimore, Maryland.

SNOW, DR. LAETITIA M., Wellesley College, Wellesley, Massachusetts.

SOLLMAN, DR. TORALD, Western Reserve University, Cleveland, Ohio.

SONNEBORN, DR. T. M., Johns Hopkins University, Baltimore, Maryland.

SPEIDEL, DR. CARL C., University of Virginia, University, Virginia.

SPENCER, DR. W. P., Department of Biology, College of Wooster, Wooster, Ohio.

STABLER, DR. ROBERT M., Department of Zoölogy, University of Pennsylvania, Philadelphia, Pennsylvania.

STARK, DR. MARY B., New York Homeopathic Medical College and Flower Hospital, New York City.

STEINBACH, DR. HENRY BURR, Columbia University, New York City.

STERN, DR. CURT, Department of Zoölogy, University of Rochester, Rochester, New York.

STEWART, DR. DOROTHY R., Skidmore College, Saratoga Springs, New York.

STOCKARD, PROF. C. R., Cornell University Medical College, 1300 York Avenue, New York City.

STOKEY, DR. ALMA G., Department of Botany, Mount Holyoke College, South Hadley, Massachusetts.

STRONG, PROF. O. S., College of Physicians and Surgeons, Columbia University, New York City.

STUNKARD, DR. HORACE W., New York University, University Heights, New York City.

STURTEVANT, DR. ALFRED H., California Institute of Technology, Pasadena, California.

SUMMERS, DR. FRANCIS MARION, Department of Biology, College of the City of New York, New York City.

SUMWALT, DR. MARGARET, Department of Pharmacology, University of Michigan, Ann Arbor, Michigan.

SWETT, DR. FRANCIS H., Duke University Medical School, Durham, North Carolina.

TAFT, DR. CHARLES H., JR., University of Texas Medical School, Galveston, Texas.

TASHIRO, DR. SHIRO, Medical College, University of Cincinnati, Cincinnati, Ohio.

TAYLOR, DR. WILLIAM R., University of Michigan, Ann Arbor, Michigan.

Tennent, Prof. D. H., Bryn Mawr College, Bryn Mawr, Pennsylvania.

TeWinkel, Dr. L. E., Department of Zoölogy, Smith College, Northampton, Massachusetts.

Turner, Dr. Abby, Department of Physiology, Mount Holyoke College, South Hadley, Massachusetts.

Turner, Prof. C. L., Northwestern University, Evanston, Illinois.

Tyler, Dr. Albert, California Institute of Technology, Pasadena, California.

Uhlenhuth, Dr. Eduard, University of Maryland, School of Medicine, Baltimore, Maryland.

Unger, Dr. W. Byers, Dartmouth College, Hanover, New Hampshire.

Visscher, Dr. J. Paul, Western Reserve University, Cleveland, Ohio.

Waite, Prof. F. C., Western Reserve University Medical School, Cleveland, Ohio.

Ward, Prof. Henry B., University of Illinois, Urbana, Illinois.

Warren, Dr. Herbert S., 1405 Greywall Lane, Overbrook Hills, Pennsylvania.

Waterman, Dr. Allyn J., Department of Biology, Williams College, Williamstown, Massachusetts.

Weiss, Dr. Paul A., Department of Zoölogy, The University of Chicago, Chicago, Illinois.

Wenrich, Dr. D. H., University of Pennsylvania, Philadelphia, Pennsylvania.

Whedon, Dr. A. D., North Dakota Agricultural College, Fargo, North Dakota.

Whitaker, Dr. Douglas M., P. O. Box 2514, Stanford University, California.

White, Dr. E. Grace, Wilson College, Chambersburg, Pennsylvania.

Whiting, Dr. Phineas W., Zoölogical Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania.

Whitney, Dr. David D., University of Nebraska, Lincoln, Nebraska.

Wichterman, Dr. Ralph, Biology Department, Temple University, Philadelphia, Pennsylvania.

Wieman, Prof. H. L., University of Cincinnati, Cincinnati, Ohio.

Willier, Dr. B. H., Department of Zoölogy, University of Rochester, Rochester, New York.

Wilson, Prof. H. V., University of North Carolina, Chapel Hill, North Carolina.

Wilson, Dr. J. W., Brown University, Providence, Rhode Island.

Witschi, Prof. Emil, Department of Zöology, State University of Iowa, Iowa City, Iowa.

Wolf, Dr. Ernst, Biological Laboratories, Harvard University, Cambridge, Massachusetts.

Woodruff, Prof. L. L., Yale University, New Haven, Connecticut.

Woodward, Dr. Alvalyn E., Zoölogy Department, University of Michigan, Ann Arbor, Michigan.

Yntema, Dr. C. L., Department of Anatomy, Cornell University Medical College, 1300 York Avenue, New York City.

Young, Dr. B. P., Cornell University, Ithaca, New York.

Young, Dr. D. B., 7128 Hampden Lane, Bethesda, Maryland.

Zeleny, Dr. Charles, University of Illinois, Urbana, Illinois.

STUDIES ON THE TREMATODES OF WOODS HOLE

II. The Life Cycle of Stephanostomum tenue (Linton)[1]

W. E. MARTIN

(From DePauw University and the Marine Biological Laboratory,
Woods Hole, Massachusetts)

This paper deals with the results of a study of a member of the trematode family Acanthocolpidae obtained during the summers of 1936 and 1938 at the Marine Biological Laboratory at Woods Hole, Mass. No previous experimental work has been done on the life cycles in this family, and consequently the systematic relationships have been in question. This paper throws some light on these problems. The results obtained may be of some economic importance because the adult members of this family are parasitic in marine fishes, several of which are food fishes.

A synopsis of this work was given before the American Society of Parasitologists at the 1938 meeting at Richmond, Virginia.

Historical

Some of the members of the family Acanthocolpidae were at first assigned to the old pseudogenus, *Distomum*, and, due to the presence of spines encircling the mouth, were thought to be related to the echinostomes. Nicoll (1915) placed some of the acanthocolpids in the family Allocreadiidae because of the similarity in the arrangement of the reproductive organs in the two groups. Winfield (1929) criticizes Nicoll's classification, stating, "The Stephanochasminae should be excluded (from the Allocreadiidae) because of the Y-shaped excretory bladder, the circle of head spines, and the armed cirrus and vagina." The family name, Acanthocolpidae, was created by Lühe in 1909 to include trematodes whose principal diagnostic characters are: a well-developed prepharynx and pharynx, a very short esophagus, a Y-shaped excretory bladder, the ovary in front of the testes, the uterus between the ovary and the ventral sucker, the cirrus and vagina armed with spines, and the genital opening medially located anterior to the ventral sucker. At present the following seven genera are included in the family: *Stephanostomum* Looss 1899, *Dihemistephanus* Looss 1901, *Deropristis* Odhner 1902, *Acanthocolpus* Odhner 1905,

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[1] This work was made possible through the use of the laboratory facilities maintained by Purdue University at the Marine Biological Laboratory.

Acanthopsolus Lühe 1906, *Tormopsolus* Poche 1925, and *Echinostephanus* Yamaguti 1934. Because of the presence of connections between the excretory bladder and the ceca in the genus *Echinostephanus*, Yamaguti separated it from the genus *Stephanostomum*. However, McFarlane (1936) described such connections in *Stephanostomum casum* (Linton) and indications of them in *S. tristephanum*. This suggests that a more extensive and intensive study of this character is needed.

Reports of observations pertaining to the life cycles of members of this family have appeared from time to time. Lebour (1907) described a cercaria that developed in rediae in the limpet, *Patella vulgata*, which she believed to be the larval form of some member of the genus *Stephanostomum*. However, this cercaria lacked eyespots, had a long esophagus and a small sac-shaped excretory bladder, all of which were contrary to observations on the adult worms. The same author (1910) described a cercaria from *Buccinum undatum* which she thought was the larval form of *Acanthopsolus lageniformis*. This cercaria possessed eyespots and general characteristics which agreed with the structures of the adult. No experimental work was done to test the validity of her assumption. Some of the cercariae had tails while the majority did not, which, in conjunction with the absence of large glands in the body, was interpreted by Lebour to indicate that no second intermediate host was required. This seems questionable since Linton (1898), Stafford (1904), Lühe (1906), Nicoll and Small (1909), Nicoll (1910), and others have found metacercariae of this family in various species of fishes. Linton (1898) found cysts of *Distomum valdeinflatum* attached to the peritoneum of *Alutera schoepfi* and *Menidia menidia notata*. Stafford (1904) found the cysts of *Stephanochasmus histrix* on the fins of *Pseudopleuronectes americanus*. Lühe (1906) found *Stephanochasmus ceylonicus* encysted in the subcutaneous tissue of *Narcine timlei* taken off Dutch Bay, Ceylon. Lebour (1907) reported *Stephanochasmus metacercariae*, probably *S. baccatus*, under the skin of the dab, witch, and long rough dab. Nicoll and Small (1909) discovered the cysts of *Stephanochasmus baccatus* under the skin of *Pleuronectes limanda*. They state, "It is not at all improbable that the cercariae of *S. caducus*, *S. triglae*, and *S. baccatus* are all to be found encysted in young pleuronectid fishes." Nicoll (1910) reported finding cysts of *S. baccatus* in *Drepanopsetta platessoides*. Yamaguti (1934) found cysts of *Stephanochasmus sp.* with 46 collar spines in *Lotella physis* and *Engraulis japonica*, *S. sp.* with 36 collar spines in *Argentina kagoshimae*, and *S. sp.* with 54 collar spines in *Bothrocara zesta* and *Furcimarius sp.* He also found *Echinostephanus* *sp.* with 40 collar spines encysted in the flesh of *Argentina kagoshimae.* The same author (1937) reported *Stephanochasmus bicoronatus* cysts in the body cavity of *Acanthogobius hasta* and on the gills of *Sciaena sp.* and *Taenioides lacepedi; Echinostephanus hispidis* cysts in the flesh of *Pseudorhombus pentophthalmus* and *Neopercis sexfasciatus* and *Tormopsolus* larvae encysted near the gills of *Leiognathus rivulata.*

Material and Methods

The snail, *Nassa obsoleta*, which serves as the first intermediate host, *Menidia menidia notata* the second intermediate host, and the puffer, *Spheroides maculatus*, which serves as the experimental definitive host, were all collected in the vicinity of Woods Hole. Naturally infected snails were used as sources of cercariae. Some *Menidia* and *Spheroides* were used for experimental feedings while others were retained as controls.

Living material was used in the study of many of the cercarial structures. Bouin's solution and a saturated aqueous solution of mercuric chloride were used as fixatives. Mayer's paracarmine was used to stain toto mounts, while sectioned material was stained with Ehrlich's hematoxylin. Infected snails, isolated in finger bowls filled with sea water, furnished a plentiful supply of cercariae for the experimental infection of *Menidia.*

Observations and Descriptions

The life cycle of *Stephanostomum tenue* involves the production of rediae and cercariae in the digestive gland of the marine snail, *Nassa obsoleta*, the development of the metacercariae in cysts in the liver of the small fish, *Menidia menidia notata*, and the maturation of the worm in the intestine of the puffer, *Spheroides maculatus.*

All measurements listed in this paper are expressed in millimeters.

The Redia (*Figs. 3 and 4*)

Natural infections of this trematode were found in about .4 per cent of the several thousand *Nassa obsoleta* under observation. Some increase in the number of infected snails in the latter part of the summer was noted, which may be correlated with the migratory habits of the hosts of the adult worms. The redia is an elongate, saccular structure with a pharynx and short rhabdocoel gut. The length of the gut, however, varies with age since it is nearly two-thirds the length of the very young redia (Fig. 4). The young redia also exhibits marked motility. The length of the redia varies from 0.14 to 0.66 with an average of about 0.5; the width varies from 0.03 to 0.14 with an average of about 0.10. The pharynx varies from about 0.025 long by 0.028 wide to 0.052 long by 0.029 wide. The number of germ balls and cercariae per redia varies from 0 to 14 for the former and 0 to 5 for the latter. No ambulatory processes were present and no birth pore was observed.

The Cercaria (Fig. 1)

The cercaria is of the ophthalmoxiphidio type with a simple tail. In swimming the tail is lashed back and forth while the body is held almost straight. In finger bowls of sea water the cercariae swim about for a short time and then settle to the bottom to which they adhere by the tips of their tails. No special glandular bodies were found in the distal region of the tail that might account for this adhesive action. This behavior may be of importance in the completion of the life cycle since the cercariae may become attached to food particles and may be eaten by fishes. The cercaria exhibits a positive response to light.

The cuticula of the body is spinous with larger spines on the anterior end. In addition to the spines there are seven or eight setae projecting from each side of the body. These are irregularly spaced along the entire body length. The oral sucker bears two rows (of 21 each) of alternating large spines about 0.005 long. These spines are easily detached under even slight cover-glass pressure. The body length varies with the degree of contraction from 0.145 to 0.38 with an average of 0.24, while the body width varies from 0.045 to 0.086 with an average of 0.064. The tail averages about 0.183 long by 0.031 wide. The oral sucker averages about 0.031 long by 0.030 wide while the ventral sucker averages about 0.033 long by 0.030 wide. The ventral sucker bears two rows of small papillae with about 65 papillae in each row. Projecting anteriorly above the oral sucker there is a simple spear-shaped stylet about 0.014 long. The mouth

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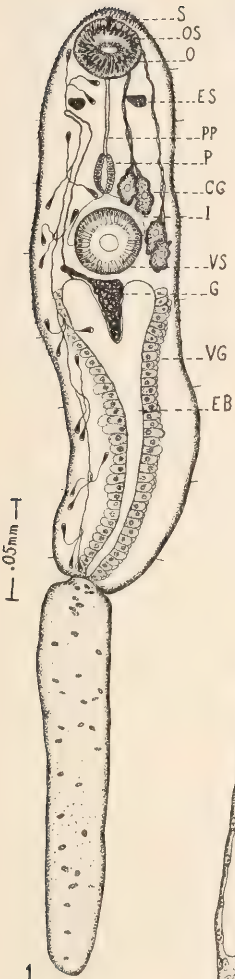
EXPLANATION OF PLATE

All drawings were made with the aid of a camera lucida. Abbreviations used: *CG*, cephalic gland; *EB*, excretory bladder; *EG*, esophageal gland; *ES*, eyespot; *G*, genital anlage; *GB*, germ ball; *GP*, genital pore; *I*, intestine; *O*, oral sucker; *OS*, oral spines; *OV*, ovary; *P*, pharynx; *PP*, prepharynx; *S*, stylet; *T*, testes; *V*, vitellaria; *VG*, vesicular gland; *VS*, ventral sucker.

FIG. 1. Ventral view of cercaria.
FIG. 2. Stylet of cercaria.
FIG. 3. Redia with germ balls and cercaria.
FIG. 4. Young redia showing elongate intestine.
FIG. 5. Metacercaria.
FIG. 6. Adult.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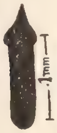
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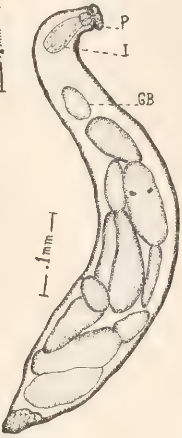
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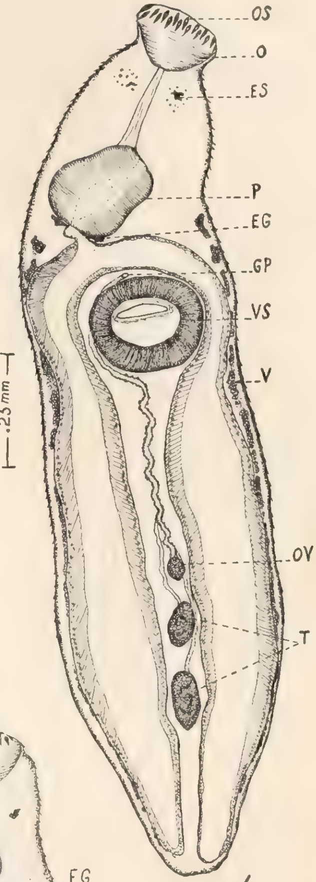
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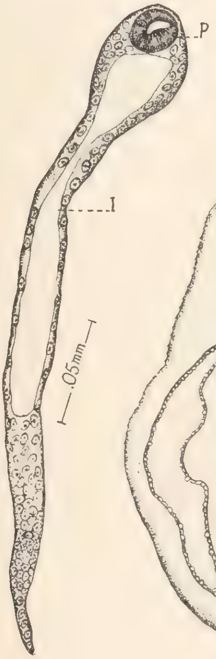
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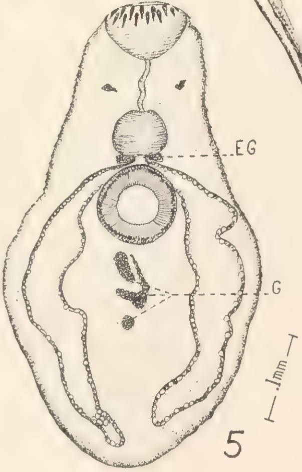
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opens into a long narrow prepharynx approximately 0.038 long. The pharynx is subglobular and measures about 0.012 in length and width. The esophagus is extremely short. The rudimentary intestine branches just anterior to the ventral sucker and the branches do not extend beyond this organ. Two conspicuous eyespots are located, one on each side of the body, near the oral sucker. Four cephalic glands are located on each side of the body immediately lateral and anterior to the ventral sucker. On each side of the body the ducts from two glands pass anteriad median to the eyespot while the ducts from the other two glands pass anteriad lateral to the eyespot. The ducts of all four glands open to the exterior at the anterior end of the body. Other glands include numerous vesicular glands along the wall of the excretory bladder. The weakly Y-shaped excretory bladder extends almost to the ventral sucker. In some specimens the anterior wall of the bladder has a scalloped appearance. The main collecting ducts arise from the anterior margin of the excretory bladder and pass anteriad to the level of the eyespots where they bend on themselves and pass posteriad to supply both sides of the body. The flame cells are in seven groups of threes, with the first group given off just after the main duct bends posteriorly at the eyespot level. The other groups are given off at intervals along the side of the body.

The reproductive system is represented by a mass of deeply staining cells located just posterior to the ventral sucker and partially surrounded by the anterior wall of the excretory bladder.

The Metacercaria (*Fig. 5*)

The cercariae are taken into the digestive tract of the second intermediate host, *Menidia menidia notata*, where they work their way through the intestinal wall and encyst in the liver or mesenteries. No cercariae were observed to penetrate the bodies of the fishes through the skin. The metacercaria increases to several times the size of the cercaria. The 42 collar spines also increase in size until they are approximately 0.050 long. The eyespots and the glandular cells surrounding the excretory bladder undergo disintegration. There is a marked increase in the size of the pharynx. The branches of the intestine develop until they reach to near the posterior end of the body. The metacercaria is held within a rather tough, loose encystment sac.

The Adult (*Fig. 6*)

Nearly mature adult worms were obtained by feeding pieces of *Menidia* liver containing metacercariae to young puffers, *Spheroides maculatus*. The puffers were examined about two weeks after the initial feeding and the worms were recovered from the intestine. Remnants of the eyespots were still present. The oral spines were the same in number and approximately of the same size as in the metacercaria. The relative proportions of the suckers and the pharynx were about the same as in the metacercaria. Advances in development over the conditions found in the metacercaria are: the differentiated testes and ovary located in the posterior one-third of the body, the reproductive tubes extending from these organs to the genital pore located on the mid-ventral side of the body immediately anterior to the ventral sucker, and the small clusters of vitelline cells extending along the sides of the body from the posterior end of the pharynx to near the posterior end of the body. Complete functional maturity of the reproductive systems had not been attained since no eggs had been produced.

The following measurements and description are based on but a few worms so that the range of variation is probably less than would be found with a larger number of individuals. Body length 1.9 to 2.2, width 0.5; oral sucker 0.13 long by 0.18 wide; ventral sucker 0.22 long by 0.25 wide; prepharynx from 0.19 to 0.31 in length by about 0.015 in width near the oral sucker to 0.031 at its widest point near the pharynx; pharynx about 0.22 long by 0.16 to 0.18 in width; esophagus 0.04 to 0.07 long; ovary about 0.057 long by 0.03 to 0.038 wide; anterior testis 0.10 to 0.136 long by 0.04 to 0.07 wide, posterior testis 0.09 to 0.14 long by 0.04 to 0.07 wide.

Linton (1898) described *Distomum tenue* from the rectum of the striped bass, *Roccus lineatus*, collected at Woods Hole. The description he gave, with measurements in millimeters, is as follows: oral spines 0.051 long by 0.018 wide at base; esophagus 0.44 long by 0.34 wide (he undoubtedly has used the term esophagus for the pharynx); vitellaria voluminous, peripheral in the posterior region; genital aperture immediately in front of the ventral sucker; ova not numerous and comparatively large, lying close behind the ventral sucker; ova length 0.088, width 0.044; body length 2.9, width 0.28; diameter of oral sucker 0.26, of ventral sucker 0.38.

Discussion and Conclusions

Most descriptions of the adult members of this family show them to have remnants of eyespots. This may indicate that the family represents a fairly compact, closely related group. When the excretory bladder is mentioned at all in the descriptions of species, it is described as Y-shaped. However, in my study of living specimens of *Deropristis inflata*, a simple tubular or sac-shaped bladder was found.

There is very little in the literature on the rest of the excretory system although Pratt (1916) in his description of *Stephanochasmus casum* showed that the main collecting tubes pass anteriad to near the level of the eyespots without giving off secondary tubes.

The arrangement of the reproductive organs in the family Acanthocolpidae, as was pointed out by Nicoll (1915), is similar to the arrangement of these organs in the family Allocreadiidae. There is also some suggestion of similarity in the excretory systems of these two groups. In addition, the members of both families are primarily parasites of fishes. This suggests a rather close relationship between the two families. However, the elucidation of the life cycles of other genera is needed before a positive statement can be made.

The family Acanthocolpidae seems to be cosmopolitan in distribution since some of its members have been found in European, Greenland, North American, Japanese, and Ceylonese waters.

There has been some confusion in the literature concerning the generic name *Stephanostomum*. This confusion resulted from Looss' first (1899) naming the genus *Stephanostomum* and then changing it to *Stephanochasmus* (1900) because of its similarity to the genus *Stephanostoma* Danielson and Koren, a genus of Gephyrean worms.

SUMMARY

It was found that the life cycle of *Stephanostomum tenue* involves the development of rediae and cercariae in the marine snail, *Nassa obsoleta*, the utilization of the small fish, *Menidia menidia notata*, as the second intermediate host, and the development of the adult worm in the intestine of the puffer, *Spheroides maculatus*. Although the puffer may serve as the experimental definitive host, the striped bass, *Roccus lineatus*, is probably a natural one.

About .4 per cent of the *Nassa obsoleta* observed were infected with this parasite.

The excretory system of the cercaria is represented by the formula $2[3+3+3+3+3+3+3]$.

The arrangement of the reproductive organs, some similarity in the excretory systems, and the fact that fishes serve as hosts to the adult worms suggest an affinity of the Acanthocolpidae to the family Allocreadiidae.

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AN HERMAPHRODITE ARBACIA

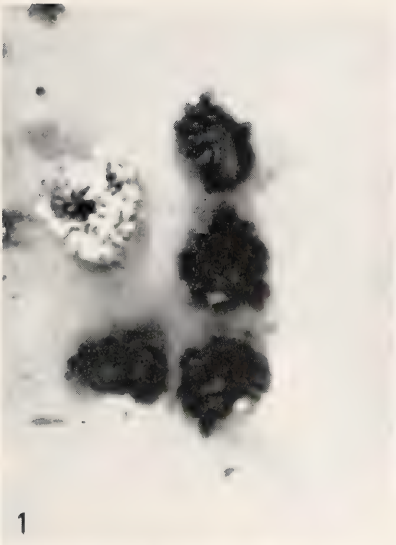
ETHEL BROWNE HARVEY

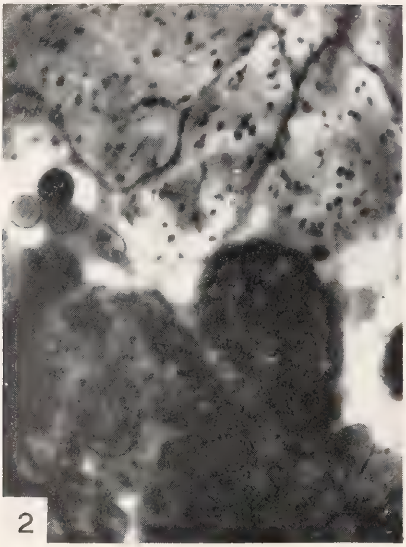
(From the Biological Laboratory, Princeton University, and the Marine Biological Laboratory, Woods Hole, Mass.)

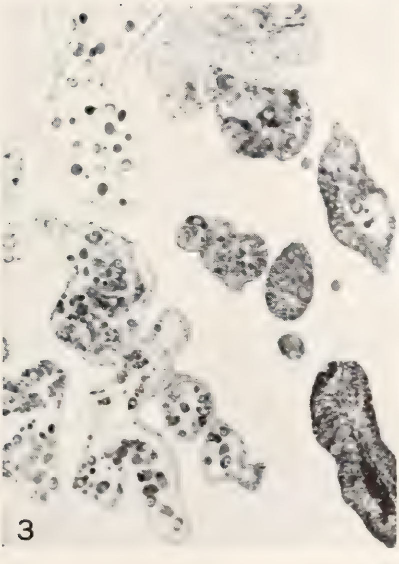
Among the many thousands of *Arbacia punctulata* opened in the course of ten summers at Woods Hole, and many hundreds of *Arbacia pustulosa*, *Sphaerechinus granularis*, *Paracentrotus lividus* and *Parechinus microtuberculatus* opened during several springs at Naples, and many hundreds of *Strongylocentrotus dröbachiensis*, from Maine, I observed last summer for the first time an hermaphrodite sea urchin, an *Arbacia punctulata*, opened on July 4, 1938. One other case of hermaphroditism in *Arbacia punctulata* has been described by Shapiro (1935); it was found late in the season of 1934 at Woods Hole. His animal had four testes and one ovary. It was fertile *inter se*, and all the eggs formed fertilization membranes, but the cleavages were delayed and abnormal. Many blastulae were obtained and 30 per cent gave rise to gastrulae; there was apparently no further development. James Gray (1921) described a *Strongylocentrotus lividus* in which three of the gonads were completely female, another almost completely so and the fifth contained both eggs and sperm which were fertile *inter se;* development of the eggs is not described. Gadd (1907) described a case of hermaphroditism in *Strongylocentrotus dröbachiensis* at the Mourmanschen Biological Station which had four female gonads and one male, but he does not give any details. The above are the only recorded cases of hermaphroditism in sea urchins, and it is indeed a rare phenomenon.

The hermaphrodite *Arbacia* which I found last summer was quite normal in external appearance and of average size. On removing the ventral portion of the shell, as usual in preparing the eggs, the gonads looked normal except that four were red ovaries and the fifth a white testis with sperm oozing out. Photograph 1 is of the five gonads immediately after removal to sea water. Microscopic observation of the living gonads showed that none of them was entirely male or female. The ovaries had a few tubules containing sperm and the testis contained some ova in various stages of development; that is, the gonads were really ovo-testes but predominantly female or male. A portion of a gonad, living and unstained, is shown in Photograph 2; the ovarian tubules are dark (from the red pigment) and the testis tubules are light with scattered pigment spots; a few eggs have been liberated and lie free in the space between the tubules. A stained

EXPLANATION OF PLATES







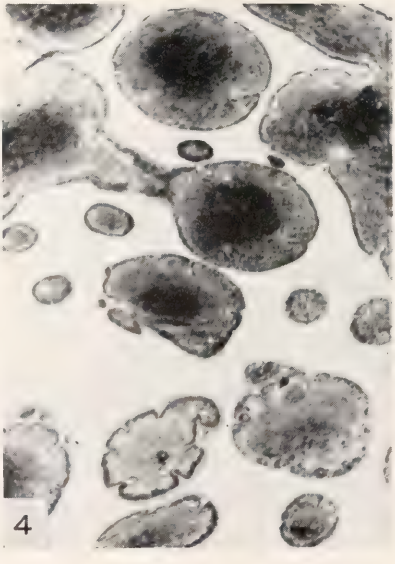


Plate I

Photograph 1. Gonads of the hermaphrodite *Arbacia*, immediately after removal from the shell; one testis (white) and four ovaries (black). Note the small piece of testis (white) at edge of the lower right ovary.

Photograph 2. Part of a living gonad, showing testis tubules (white with pigment spots) and ovarian tubules (black) containing eggs, as seen under the microscope. A few eggs are seen free in the space between the tubules.

Photograph 3. Prepared stained section of a gonad predominantly female containing eggs in various stages of maturity. One testis tubule is seen at lower right.

Photograph 4. Prepared stained section of a gonad, predominantly male, containing mostly ripe sperm. One ovarian tubule is seen at lower right.

section of a predominantly female gonad is shown in Photograph 3; all the tubules are filled with eggs in various stages of development except the lower right which is mostly testis. Photograph 4 is a section of the predominantly male gonad; the tubules are all filled with sperm except one at the lower right which contains eggs. Photograph 5 is a section of a predominantly female gonad showing greater detail. Sections of normal ovaries and testes are exactly like these except that there is no mixture. As far as I could tell, especially from a study of the living gonads, the eggs and sperm in the hermaphrodite gonad are separate in the small tubules, and do not lie together without any partition. The eggs are not fertilized until they have been liberated from the tubules into the sea water, probably because the sperm are not motile until in sea water. As soon as the eggs have poured out from the tubules into the sea water, they are immediately fertilized by the sperm which have poured into the sea water and become active. At any one time, therefore, the fertilized eggs are found in various stages of development.

The eggs of the hermaphrodite are perfectly fertile with its own sperm. Normal fertilization membranes are formed, first cleavage takes place normally and at the normal time, and the later cleavages also, and practically all the eggs develop. The only unusual phenomenon was the occurrence of giant eggs. These were about 1 per cent of the total and were all of the same size, 96 μ in diameter, giving a volume of 463,000 μ^3 whereas the normal egg has a diameter of 74 μ and a volume of 212,000 μ^3; the giant eggs are approximately twice the normal volume. The origin of the giant eggs is not known, but they do not arise from fusion of ripe eggs since giant immature eggs also occur. I have found similar giant eggs in other *Arbacias* but very rarely, and I have also found in another *Arbacia* normal-sized eggs with giant nuclei. These nuclei measured 25.6 μ in diameter giving a volume of 8,785 μ^3, whereas the normal nucleus measures 11.5 μ in diameter, giving a volume of 796 μ^3; the giant nuclei are thus about eleven times the normal volume.

Eggs in late cleavages (3½ hours, and less, after fertilization at

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PHOTOGRAPH 5. Prepared stained section of a gonad under higher magnification to show greater detail. The gonad was predominantly female, but the portion photographed predominantly male.

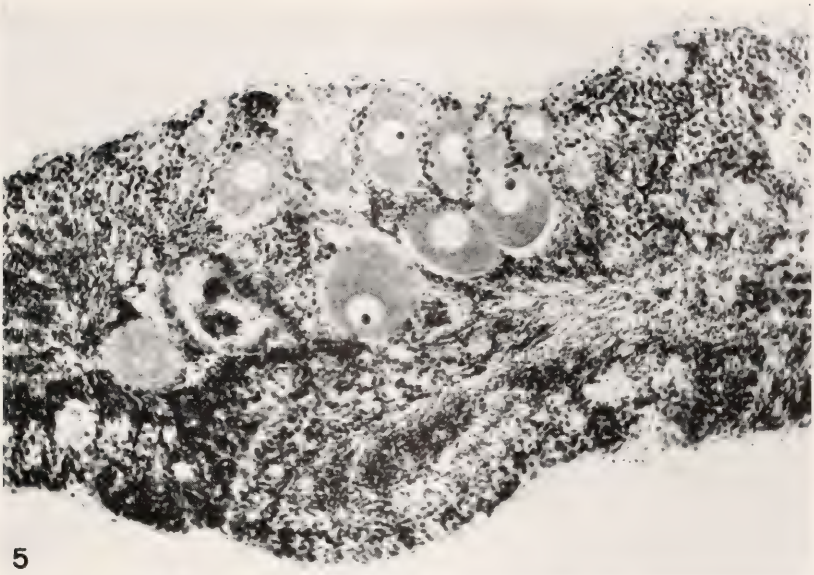
PHOTOGRAPH 6. Self-fertilized eggs 3½ hours (21° C.) after opening the animal. Most of the eggs are in late cleavage stages, but some are not so far advanced since they have come from the tubules and been fertilized later than the others. Note the giant eggs, also developing normally.

PHOTOGRAPHS 7–9. Normal development of self-fertilized eggs.

PHOTOGRAPH 7. Very early pluteus, self-fertilized, 31 hours old.

PHOTOGRAPH 8. Pluteus, self-fertilized, 35 hours.

PHOTOGRAPH 9. Pluteus, self-fertilized, 48 hours.



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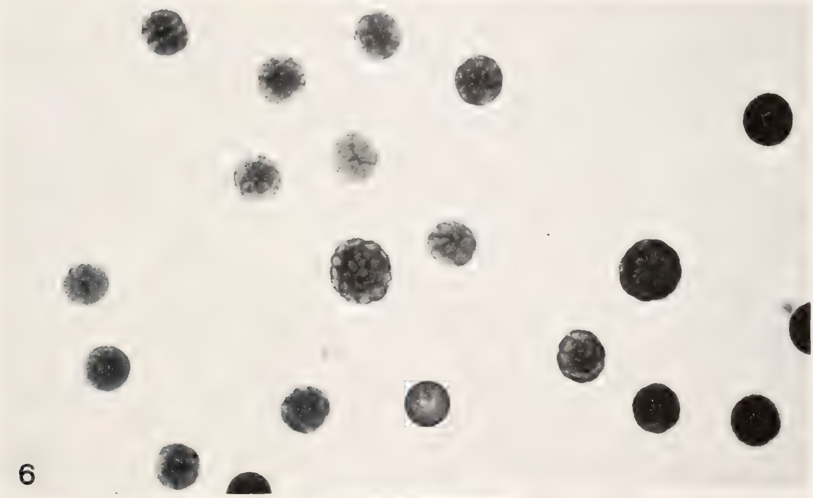

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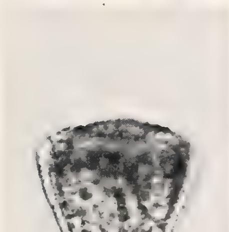

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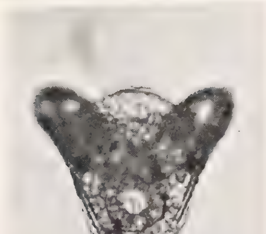

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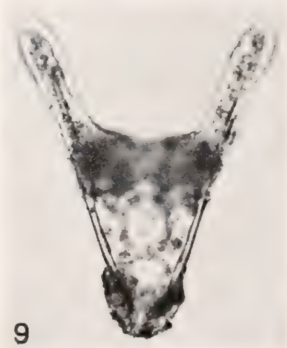

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Plate II

21° C.) are shown in Photograph 6, and one may observe here the giant eggs. The eggs, including the giant ones, develop quite normally and become swimming blastulae at the normal time, 9 hours after fertilization. The blastulae develop into perfectly normal plutei (Photographs 7–9), and these were kept for nine days. The plutei from the giant eggs were indistinguishable from the others which vary greatly in size according to age.

The sperm were perfectly normal in fertilizing other eggs as well as the hermaphrodite eggs (98 per cent), and the eggs from the hermaphrodite could be fertilized perfectly well by sperm from another sea urchin. This latter fact was ascertained by putting a small part of an ovary into fresh water for about ½ minute to kill the sperm on the outside; then the ovary was transferred to sea water. After an hour, only 1 per cent of the eggs shed were fertilized (by a few sperm liberated from the ovotestis after washing). But when the shed eggs were transferred to sea water containing sperm from another animal, 98 per cent were fertilized. The fertilization was therefore made by the sperm of the normal animal. These eggs developed quite normally. The hermaphrodite animal is therefore fertile with other males and females as well as *inter se*.

I think this the first recorded case in which the eggs of an hermaphrodite sea urchin, self-fertilized, developed absolutely normally to perfect plutei.

SUMMARY

1. A rare case of perfect functional hermaphroditism in the sea urchin *Arbacia punctulata* is described. There were four red gonads predominantly female and one white gonad predominantly male; there were a few tubules of the opposite sex in all the gonads.

2. Fertilization occurred as soon as the sexual products were liberated in sea water.

3. The development of the self-fertilized eggs was absolutely normal, in time and morphology, and normal plutei were raised, nine days old.

4. There occurred about 1 per cent of giant eggs; these were twice the normal volume, and they also developed normally.

5. Both the eggs and the sperm also functioned perfectly normally with other normal males and females.

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KARYOKINESIS DURING CLEAVAGE OF THE ZEBRA FISH BRACHYDANIO RERIO

EDWARD C. ROOSEN-RUNGE

(From the Department of Biology, Brown University)

INTRODUCTION

The results presented in this paper have been obtained in the course of a comprehensive study on the periodicity of cell division and mitotic rate during development. A discussion of these results, confined to observations on teleosts, is a necessary preliminary to the more complete investigation, with history and literature, to be presented later.

To obtain a definite picture of the rôle of cell division in development, it is necessary to determine not only the number of mitoses which occur at given times and at given places, but also the duration of a single mitosis and the manner in which it proceeds in different stages of development. In spite of many careful investigations on the rate of mitosis, the duration of mitosis at different periods of development has not been sufficiently determined.

The most successful method in investigating the rôle of cell division in development has been that of Richards (1935) and others who tried to determine mitotic activity by means of a mitotic index or the percentage number of dividing cells. However, it is not possible to tell by this method whether mitoses occur at periodic cycles or are evenly distributed, so that the counting at any time will actually furnish a figure which approximates the average mitotic rate. The present paper deals with the manner and duration of mitosis only during cleavage.

The egg of the zebra fish (*Brachydanio rerio*), recently described as a favorable laboratory subject (Roosen-Runge, 1938), is especially adapted to this study, because of its rapid development, its transparency, and more particularly because the cell nuclei can be easily observed in the living egg. Three lines of investigation will be described in this paper, namely, (1) the morphology of the living and of the fixed nuclei; (2) the duration of divisions and of mitotic phases; and (3) their reaction to temperature changes.

MATERIALS AND METHODS

Information concerning the propagation and raising of the eggs of the zebra fish may be found in an earlier paper (Roosen-Runge). For observation of the living egg, a slide with a covering about 1 mm. thick of a mixture of bee's wax and paraffine was used. A hole, the diameter of the egg, was then cut through the layer of wax in order to let the light come through, with a glass ring, 22 mm. wide and 9 mm. high, added to prevent currents from moving the egg. The slide was immersed in water in a large dish of about 150 cc. capacity, to insure an abundant oxygen supply. The egg was then oriented in the hole and all observation carried on, with the slide so immersed, by means of a water-immersion lens (Zeiss, × 40), having sufficient depth of focus to make visible the cells inside the cell membrane. Although the use of an oil-immersion lens is also feasible, it is only useful to check up on details which on the whole can be seen just as clearly with the water-immersion lens.

The temperature was regulated with an ordinary desk lamp shining from varying distances upon the observation dish. This simple device proved sufficient to keep the temperature constant within the range of half a degree Centigrade, since the amount of water in which the egg was kept, being fairly large, made it possible to control the temperature almost continuously during the period of development. Thus the eggs continued to develop under the microscope without the least sign of disturbance from the beginning of the second to the end of the tenth cleavage, that is, for a period of about three hours.

Bouin's solution was used for fixation. The egg membrane and in most cases the yolk were removed after fixation, for it is then quite easy to tear off the membrane from the hardened egg and to remove the brittle yolk. Dioxan or alcohol + benzol was used for dehydration, but the former is the simpler method and, therefore, to be preferred. All sections were stained in Heidenhain's haematoxylin and cut 6 or 8 microns in thickness.

Morphology of the Nuclei

It is impossible to study the nucleus in the living egg before the first cleavage since the delicate structure is then hidden by coarse granules which are whirled up at the base of the cell by the streaming of the protoplasm into the blastodisc. During the first cleavage the streaming still continues, offering some difficulties to the observer. Accurate observation of the nucleus becomes possible only when the cytoplasm clears at the end of the first cleavage. The two nuclei appear as ovals with a longitudinal axis of approximately 18 μ. The outlines are fine and smooth. Two or three, sometimes more, very delicate curved lines divide the nucleus into several sections (Fig. 1). The first signs of mitosis are the swelling of the nucleus and the appearance of irregularities in its oval shape when tiny indentations can be seen at the poles which appear flattened so that the nucleus assumes a barrel-like shape. Short rays which point toward the center of the nucleus seem to radiate from the depths of the indentations. Very often the nucleus appears to be divided lengthwise into halves by a fine channel which is filled with some substance a shade darker than the nuclear sap.

In the living egg the appearance of the indentations marks the beginning of a very rapid disintegration of the nuclear membrane. The whole circumference appears strongly wrinkled and rapidly fades out, together with the partition lines inside of the nucleus. In a short time no traces of nuclear structures are left. By watching very closely, one can for a moment fancy where the nucleus has been, because this area appears somewhat lighter and free from the tiny granules which are a part of the cytoplasm throughout the cell. Before nuclear structures become visible again, the cell almost completes its division. The changes in the cytoplasm and the shape of the cell during mitosis have already been described (Roosen-Runge, 1938).

Sometime after the furrow has completely cut through, there appears in the center of each daughter cell a group of tiny dark granules. These granules represent the chromosomes. They swell, become lighter, and finally appear as little circles or vesicles with very distinct outlines. The vesicles go on swelling rapidly until they come into contact with each other, eventually forming one body with a common but irregularly curved contour. The outlines of the individual vesicles remain visible for a time, some of them fading out finally, while others do not disappear until the breakdown of the nuclear membrane in the next prophase.

Observations on the living nuclei confirm some of the results obtained from sectioned material. The outstanding feature in the karyokinesis of the teleost blastomeres is the formation of chromosomal vesicles during the telophase. These chromosomal vesicles are quite commonly found in early development and are supposed to persist through the interkinetic phase into the prophase. This interpretation has been made very probable by A. Richards (1917) and B. G. Smith (1929) from the study of sections. It can be proved by the study of living nuclei, in which some of the walls of the vesicles can actually be seen to persist in the interphase nucleus. Some of the walls, however, do not remain visible, but this seems to be due to their thinning out and not to their complete disappearance, since the sections also show some partitions, very dark and distinct, while others are delicate and inconspicuous. In many instances the sectioned nuclei can be seen divided into halves, inside of which the vesicles are visible. The halves are separated by a gap, apparently filled with cytoplasm, which corresponds to the observations on the living nuclei. The halves represent the paternal and the maternal parts of the chromosome set, as first described by Moenkhaus (1904) in teleost hybrids, and by many early workers on other forms.

How the vesicles arise from the anaphase chromosomes and how the chromosomes are formed from the vesicles in the prophase, cannot be determined accurately from fixed material, nor do observations of the living nuclei solve any of these problems. Richards (1917) concluded that the vesicles are formed by a swelling of the chromosomes so that finally the walls contain the chromatin material and enclose a space "filled in from the fluid portion of protoplasm." Smith (1929), on the other hand, studied the karyokinesis in *Cryptobranchus* eggs and found that the vesicular membrane was of cytoplasmic origin, developed under the influence of the chromosome within. Pictures like those of Smith certainly cannot be seen in sections of either *Fundulus* or *Brachydanio* eggs. The study of living nuclei only confirms the impression that the chromosomes actually swell during the telophase and that the vesicular wall represents the surface of the chromosome rather than a structure formed *de novo* from the cytoplasm. My own material does not show some of the details as distinctly as they appear (according to Richards) in *Fundulus*, although the formation of the vesicles and their persistence through the interphase could be clearly seen in the sections as well as in the living egg. Nevertheless, the behavior of the chromosome material still remained puzzling. That the reader may be better able to appreciate its actual appearance, I have used photographs (see plate) rather than drawings, as Richards and others have done. Attempts at drawing present possibly too great a temptation to express a prejudiced interpretation not justified by the actual material.

The prophase stage in the karyokinesis of the living blastomere has already been described. The appearance of the nucleus as a whole corresponds very well with the observations of the sections. Because of the rapidity with which the chromosomes reappear and arrange themselves, only a few figures in these phases will be found in material fixed at random. However, by closely watching the living nuclei and taking into account the time necessary for sufficient penetration of the fixing fluid to arrest the mitosis (about half a minute for Bouin's fluid), it is possible to fix material in any desired stage. It can then be seen that the individual chromosomes become clearly visible only immediately before the breakdown of the nuclear membrane. They seem to begin

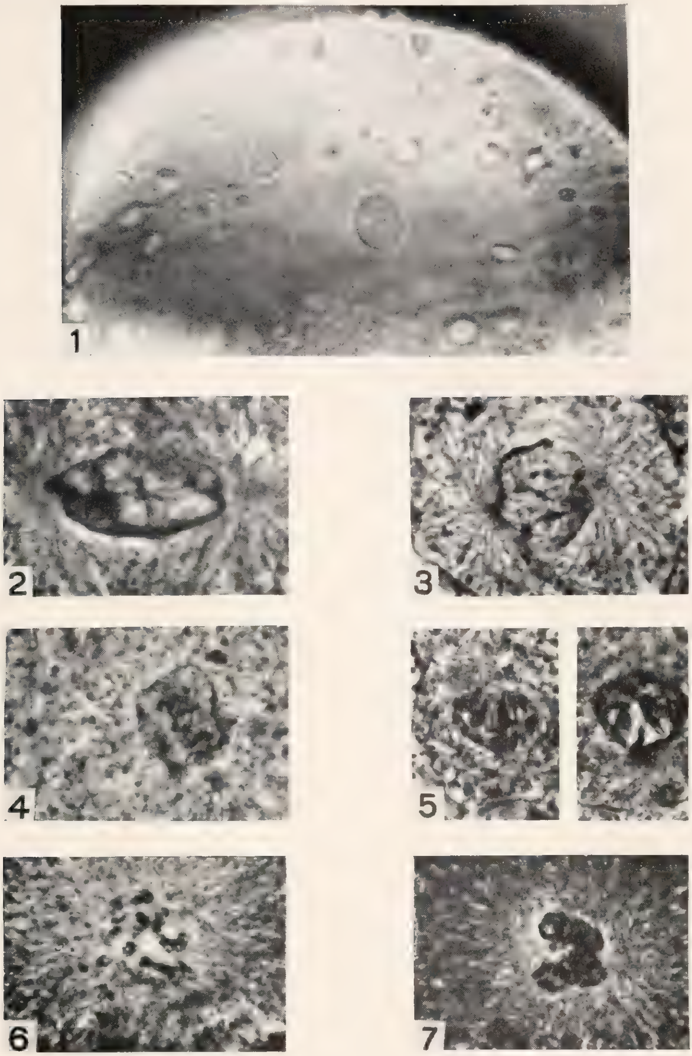

PLATE I

EXPLANATION OF FIGURES

FIG. 1. One of the eight blastomeres of a zebra fish egg, living. Nucleus with a few partition lines within, in the center. × 500.

FIGS. 2–7. Nuclei, fixed in Bouin's, Heidenhain's haematoxylin, 6–8μ. × 1100. The different sizes of the nuclei are due to their belonging to different cleavage stages.

FIG. 2. Prophase in the beginning. Vesicles still visible. Indentations at the poles.

FIG. 3. Advanced prophase, chromosomes appearing.

FIG. 4. Chromosomes forming metaphase plate. Outline of nucleus still visible.

FIG. 5. Nuclei from cells after the twelfth cleavage, showing typical spireme formation in the prophase.

FIG. 6. Early telophase. Formation of chromosomal vesicles.

FIG. 7. Late telophase. Vesicles in contact. Paternal and maternal half of chromosomes apparently separate.

the arrangement into a plate while still inside of the membrane (Fig. 4). This fact has been confirmed by observations on the living eggs of another teleost, *Epiplaty chaperi*, in which the chromosomes are somewhat more easily discernible in life. Directly after the breakdown of the nuclear membrane the chromosomes can be seen arranged in a metaphase plate but very soon afterwards they begin separating. This observation shows that the disappearance of the membrane actually occurs relatively late. Individual chromosomes inside the separate vesicles, as pictured by Richards, could not be found in sections of the zebra fish egg.

The Duration of Cell Division

The absolute duration of cell division varies tremendously in different animals, and in different cells of the same animal, despite the fact that karyokinesis is supposed to occur essentially in the same way in all of them. The duration of mitosis is characteristic for the different kinds of cells. It can only be measured accurately through the direct observation of living material. The relative time of mitotic phases has been estimated by using the percentage number of cells active in the different stages, but in many cases, as will be pointed out later, this method is very erroneous. It seems, therefore, that direct observation is the safest method for determining the relative intervals in cell division.

The most considerable error in measuring the duration of cell division in life arises from the difficulty in finding any definite point of departure. Neither the beginning of the prophase nor the last stage of the telophase can be defined accurately, so that only a very few events are established sharply enough to serve as marks by which stages may be measured. In the blastomeres of the zebra fish the swelling of the nucleus at the beginning of the prophase, the breakdown of the nucleus, the appearance of the furrow, the completion of the furrow, the first appearance of the chromosomes in the telophase, and finally the completion of the rounded nucleus, furnish seven criteria of very different value. The time of the formation of the furrow, which means the division of the cytoplasm, can only be used indirectly for the determination of karyokinetic stages, although it may serve to subdivide the interval in which nuclear structures cannot be observed at all. The moment when the nucleus seems completely rounded and smoothly outlined is almost impossible to define, and its determination involves a considerable error. The swelling of the nucleus in the early prophase is also difficult to observe, but it is possible to determine its approximate beginning somewhat better with the aid of a micrometer eye-piece, the scale of which will permit accurate observation of small changes in size. The reappearance of the chromosomes as tiny granules in the telophase is an event more easily determined. Under favorable conditions it is quite possible to watch the optically empty central area of the cell and to see the chromosomes become visible. I estimate the possible error under optimal conditions to be not more than 30 seconds or 3 per cent of the whole time of cleavage. By far the best mark, because of its rapidity of occurrence, is the breakdown of the nucleus. The nuclear membrane not only disappears in from 15 to 30 seconds, but the onset of this event is foreshadowed by a series of preparatory events, namely, the swelling of the nucleus and the wrinkling of the membrane, which makes it possible to predict the time of breakdown quite accurately. The error in determining the precise time of this occurrence is certainly not greater than 15 seconds, which is about 1.5 per cent of the whole time of cleavage. We have thus found two marks which seem reliable, because their errors can be estimated with considerable accuracy at only 1.5 to 3 per cent of the entire duration of cleavage. All other marks certainly have a higher error in determination, and if they are to be used for an estimate of the duration of the mitotic phases, this uncertainty has to be kept in mind.

The time for each cleavage from the first to the tenth is almost the same in different eggs, provided that a constant temperature is maintained and the oxygen supply is sufficient. During the process of cleavage the cell divisions follow each other without a typical resting stage, therefore the cleavage time was measured from the breakdown of the nucleus to the breakdown of the daughter nuclei. In Table I the results are compared with those of Jordan and Eycleshymer (1894) on amphibian blastomeres. The numbers concerning the zebra fish egg are all averages of at least 10 eggs. It can be seen from Table I that in every species the divisions show a characteristic duration. In four of the six animals the divisions show a trend towards acceleration before they finally begin to slow down. (The more complicated curve for the *Amblystoma* egg cannot be discussed here.) The turning point for this trend comes at different times. In the egg of the zebra fish the acceleration is at its height during the fifth cleavage. It seems significant that this is the last division when only one cell layer is involved, for the sixth cleavage is horizontal and divides the blastoderm into two layers. The sixth cleavage takes a slightly longer time than the preceding division, and from then on the process of cleavage gradually becomes slower and slower. Acceleration and retardation seem to involve the whole mitotic process uniformly and not any of its phases differentially. Only during the ninth and tenth cleavages has a prolonged interkinetic phase been recorded, but as the error in determining this phase is even greater than for any other, no conclusion can be drawn from observations made at these stages of development.

TABLE I

Duration of cleavage divisions in amphibian and teleost eggs.* The times enclosed in brackets refer to individual cases and are not averages.

	Amblystoma punctatum	*Rana palustris*	*Diemectylus viridescens*	*Bufo variabilis*	*Epiplaty chaperi*	*Brachydanio rerio*
Temperature, ° C.	18	18	18	18	24	25
Duration of Cleavage Divisions						
Fertilization to first cleavage	10 hrs.?	4–5 hrs.	10 hrs.	4–5 hrs.		25 min.?
First to second cleavage	1 hour 50 min.	1 hour 15 min.	2 hrs.	1 hour 5 min.	2 hrs. 2 min.	20 min.
Second to third cleavage	1 hour 55 min.	1 hour 15 min.	1 hour 45 min.	1 hour	44 min.	19½ min.
Third to fourth cleavage	2 hrs.		1 hour 40 min.	1 hour	(43 min.)	19 min.
Fourth to fifth cleavage	1 hour 40 min.		1 hour 50 min.	1 hour	(41 min.)	18 min.
Fifth to sixth cleavage	(1 hour 35 min.)		(2 hrs. 45 min.)		(39 min.)	17½ min.
Sixth to seventh cleavage	(1 hour 25 min.)		(2 hrs. 45 min.)		(39 min.)	18½ min.
Seventh to eighth cleavage	(1 hour 25 min.)		(2 hrs.)		(40 min.)	19 min.
Eighth to ninth cleavage	(1 hour 25 min.)					20½ min.
Ninth to tenth cleavage						20 min.

* The data on amphibian eggs are taken from Jordan and Eycleshymer (1894).

In measuring the relative duration of the mitotic phases every cleavage can, of course, be observed. Most observations, however, were made during the sixth to ninth cleavages, since these stages had to be studied also for the periodicity of divisions, which will be discussed later. The arbitrary definition of the stages is obviously a matter of terminology so long as the fundamental mechanism of mitosis is not understood.

The prophase was defined as extending from the first swelling of the nucleus until the break-up of the membrane. The time from the break-up until the chromosomes reappeared was assumed to be the duration of the metaphase plus the anaphase. As to the duration of both of these phases, it can only be stated that the metaphase is much shorter than the anaphase. This is true for two reasons, namely: (1) Nuclei which were observed up to the breakdown of the membrane and then immediately fixed always showed the chromosomes already slightly apart, and (2) the very rapid passing of the metaphase as described can actually be seen in the egg of *Epiplaty chaperi*. From these observations it must be concluded that the metaphase probably takes not much longer than one minute, or about 5.5 per cent of the total division time.

TABLE II

Duration of mitosis and mitotic phases.

Material	Total duration	Prophase	Metaphase	Anaphase	Telophase	Author
	minutes	*per cent*	*per cent*	*per cent*	*per cent*	
Protozoön: *Rhagostoma schüssleri*	32.5	18.5	12.5	18.5	53.5	after Darlington
Protozoön: *Euglypha sp.*	179	22.5	14.0	3.5	60.0	after Darlington
Erythrocytes, *Triton*	150			40.0	46.5	Jolly
The same	180	19.5	12.0	19.5	50.0	Wassermann (after Jolly)
Chorioidea, cartilage in chicken, culture	35	20.0		80.0		Strangeways
Blastomeres, *Brachydanio*	16	18.5		55.5	26.0	
The same, interkinetic phase counted as telophase	18	16.5		50.0	33.5	Roosen-Runge

The telophase was measured from the appearance of the chromosomes until the nuclei were completely rounded, with only a few partitions within. It was assumed that the reappearance of the chromosomes in life actually indicated a break in the process of mitosis, inasmuch as they become visible at the moment when they begin to take up fluid and pass from more or less solid bodies into vesicles.

In Table II, some results on the relative duration of mitotic phases in various animals have been compared. They were all obtained by direct observation. Interesting data like those of Lewis and Lewis

(1917) have been omitted since they are given too inaccurately for the present purpose. They seem, however, not to be in general disagreement with the figures presented here. The significance of the data compared lies in the fact that they agree surprisingly well, in spite of the different kinds of material used by the different investigators as well as the great disparity in the observations made with relation to the total duration of mitosis and the definition of its phases.

The relative time for the prophase varies only from 18.5 to 22.5 per cent in cells as different as those of protozoa, chicken cartilage, and fish blastomeres. The reported times of the metaphase vary also only slightly. However, in both the anaphase and the telophase there is considerable variation although it is smaller in the anaphase than in the telophase.

TABLE III

The duration of cleavage divisions under different temperatures.*
The times are minutes.

Cleavage	2	3	4	5	6	7	8	9	10
23° C.						(21)			
23½		(20)	(20)			(21)			
24						20	19.5		
24½	21		19	(20)	(19)				
25		19.5	18.5	18.5	18	18.5	19	19.5	20
25½			(18)	18	17	17	18		
26			(17)				17.5	18	20
26½						(16)			

* The times enclosed in brackets refer to individual cases and are not averages.

The telophase in *Triton* erythrocytes is reported to take 50 per cent of the total time of mitosis, and in protozoa 53.5 and 60 per cent. Lewis and Lewis state that the telophase "which can be more accurately recorded than the other phases, shows a striking similarity in all types of cells and much less variation." If we take their telophase and reconstruction periods together as corresponding to the definition of the telophase used here, we find that the telophase in cultures of chicken mesenchyme and smooth muscles lasts about 50 per cent of the whole time of division, while the telophase of the zebra fish blastomeres takes only about half of this relative time, that is, 26 per cent. Even if the interkinetic phase, the delimitation of which is not at all clear, is added quite arbitrarily to the telophase, there is not more than 33.5 per cent of duration time accounted for. The certainty with which this result is obtained leads to the conclusion that the relative shortness of the telophase is actually significant for the type of karyokinesis we are dealing with, which involves the formation of chromosomal vesicles in the telophase, and has no actual "resting phase."

The effect of different temperatures on the duration of cleavage is shown in Table III. The results cover only a part of the large range of temperature which the eggs can stand. The only conclusions, therefore, which can be safely drawn are that the duration of cleavage divisions is influenced by even slight changes in temperature, but that the general trend of acceleration for the first six cleavages, and the following retardation, are practically unchanged so that the duration of mitosis may be said to be constant under constant conditions. Many investigators, however, have found the duration of mitosis varying up to several hundred per cent for the same kind of cells. Observations of cells in tissue cultures in particular have yielded results which were very inconsistent with respect to the total duration of division. In all these cases the inconsistency can be attributed only to varying conditions of nutrition, oxygen supply, and temperature. A comparison of the results given in the literature and the observations on the eggs of the zebra fish, show that under constant conditions the duration of mitosis is constant and characteristic for the different types of cells.

Discussion

The process of cleavage is characterized by continuous and often synchronous cell divisions, which frequently follow a definite pattern. In general there is no morphological differentiation during cleavage, but very often there is a segregation of different materials in different cells. At the end of the cleavage period there is a break in the development, the divisions cease to be continuous and synchronous, and the period of cell migration and arrangement begins, often together with the first histological differentiation. On the other hand, cleavage is regarded as "but a continuation . . . of that series of cell-divisions which has been going on uninterruptedly, though with periodic pauses, since the most remote antiquity. The divisions of the egg during cleavage are in all essentials of the same type as those of adult cells; such differences as may appear—e.g., the prominence of asters, the frequent asymmetry of the amphiaster, and the consequent inequality of cleavage—are of minor importance, though often interesting for analyzing the mechanism of mitosis." (E. B. Wilson, 1928, page 981.) The general conception is that cleavage divisions are dynamically somewhat different from the divisions in the older animal, but that their variation is not correlated with any essentially different mechanism. There are, however, observations which point to a difference in mechanism. Investigators of the chromosomal vesicles, which so frequently occur in the telophase of cleavage divisions, have often suspected that this particular feature of mitosis might be immediately connected with the fact that cleavage divisions go on continuously and almost without interphases.

The study of karyokinesis in zebra fish blastomeres reveals that the formation of chromosomal vesicles is obviously in itself a process of much shorter duration than the common type of telophase and, furthermore, that it represents a condition which permits of an almost immediate start of the next division without a "resting stage" and without a spireme formation in the prophase. No nucleoli are formed in this type of mitosis. All these features are characteristic only for the divisions during cleavage. About the time of the twelfth cleavage an entirely different type of mitosis appears, which shows no chromosomal vesicles in the telophase, but nucleoli and a very distinct spireme in the prophase (Fig. 5). In my material no transitional forms have been observed between these two types, though it is quite possible that a more thorough investigation may reveal such transitions.

Chromosomal vesicles have been found in the eggs of very many species and almost all classes of animals with the possible exception of birds and mammals. (A review of the literature has been given by Richards, 1917.) The suggestion seems obvious that the type of mitosis which is characterized most strikingly by the formation of chromosomal vesicles in the telophase, is due to some aspect of the division mechanism that is peculiar to the cleavage divisions. We have not yet arrived, however, at any definite conclusions concerning the possibly different dynamics involved.

SUMMARY

The nuclei in the blastomeres of *Brachydanio rerio* can be observed easily in life. They are visible in the prophase and telophase as well as in the interkinetic phase. This discovery is used (1) to confirm and consolidate the results obtained from sectioned material; (2) to fix the blastomeres in any desired mitotic phase; and (3) to determine the duration of mitosis and its phases.

The duration of mitosis and its phases under constant conditions, particularly with respect to temperature, is found to be constant for each cleavage. The time from the breakdown of 32 nuclei to the breakdown of 64 nuclei is 18 minutes at 25° C. This places the cleavage divisions of the zebra fish among the most rapid ever observed. The first six cleavages show a trend towards acceleration, the sixth being the most rapid one. From then on the speed of the divisions slows down. This trend is essentially undisturbed by changes in temperature.

The nuclear divisions during cleavage are characterized (1) by the formation of chromosomal vesicles in the telophase (some of these vesicles can frequently be seen in life to persist through the interphase); (2) by a comparatively short duration of this type of telophase; (3) by a very short, if any, true interphase; (4) by the lack of nucleoli; and (5) by the absence of a typical spireme formation in the prophase.

The very short duration of the telophase has been recorded for the first time. The other observations have been found in the cleavage divisions of a majority of the species examined. In the zebra fish egg they continue until about the twelfth cleavage, when the form of mitosis typical for the adult first appears. It is suggested that this type of mitosis is probably associated with the rapid sequence of divisions and is generally characteristic of cleavage mitoses. The most characteristic feature of this type of karyokinesis is the formation of the chromosomal vesicles, but the shortening of the interphase and telophase, and the lack of spireme formation in the prophase are also obvious.

I am indebted to Professor J. W. Wilson, Brown University, for his very valuable advice and to Professor H. E. Walter for his assistance in editing the paper.

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THE EFFECTS OF LIGHT AND TEMPERATURE ON THE MALE SEXUAL CYCLE IN FUNDULUS

SAMUEL A. MATTHEWS

(*From the Thompson Biological Laboratory, Williams College*)

Fundulus heteroclitus is a teleost fish which breeds during the late spring and early summer months. Its gonads undergo fairly definite seasonal changes, reaching their greatest weight just before the breeding season, falling off sharply just after spawning is completed in late July, then undergoing a period of slow growth until the onset of rapid maturation of germ cells prior to the next breeding season (Matthews, 1938). Several factors, acting independently or collectively, may be concerned in the control of this gonad cycle. Of these factors the pituitary gland as an internal factor and temperature and light among the external factors might reasonably be supposed to possess some degree of control. Evidence concerning the rôle that the pituitary gland plays in the sexual cycle has already been obtained (Matthews, 1939). The following experiments are concerned with the effects of light and temperature on the male sexual cycle.

From the experiments of a number of workers, particularly those of Bissonnette (see his review article, 1936) it is clear that in some birds and mammals light plays a dominant rôle and temperature a subordinate one in controlling the seasonal cycle in the gonads. The data on poikilothermous animals are not as conclusive. Clausen and Poris (1937) in the case of *Anolis* and Burger (1937) in the case of *Pseudemys* both believe that light is important in controlling the sexual cycle. Bellerby (1938), on the other hand, finds no evidence "that light is essential for the maintenance of reproductive activity in *Xenopus laevis* or that seasonal variation in light intensity or wave length plays any part in the control of the sexual cycle under natural conditions." Turner (1919), in describing seasonal changes in the spermary of the perch, pointed out that tremendous synthesis of material in the testis occurs in August when the temperature of the water has reached its peak and begun to decline, and expulsion of the sperm occurs when the temperature begins to rise. No experiments controlling the light factor were described. From his work with the stickleback Craig-Bennitt (1930) also concludes that temperature is the important factor in controlling the sexual cycle and that light is unimportant. More recently Hoover and Hubbard (1937) have shown that a gradual increase in daily illumination followed by a gradual decrease will cause brook trout (a fall breeding animal) to produce ripe eggs and mature sperm several months earlier than normal.

To determine whether or not the absence of light exerts any inhibitory influence on spermatogenesis in *Fundulus* the following two experiments were carried out. In the first, begun in December, 11 males were divided in such a way that 5 were maintained in an aquarium subjected to ordinary daylight with no night illumination and the other 6 were kept in a light-proof tank. They were fed daily with the stock food used in all experiments, consisting of dried shrimp, puppy biscuit and Mead's infant cereal, with occasional living food such as worms or *Daphnia*. These animals were killed at five different intervals over a period of 3 weeks. The average percentage of the body weight formed by the testis of the animals in the light-proof tank was 0.56 per cent, that in the other aquarium 0.37 per cent, and microscopic examination of the testes showed no significant differences in the state of activity of the two groups. In the second experiment, begun in March and extending into April, 4 males were placed in the lighted aquarium, which in this instance was illuminated at night by a 50-watt mazda bulb suspended above the tank, and 8 were kept in the dark. The animals were killed over a period of 4 weeks. The percentage of the body weight formed by the testis in the illuminated aquarium averaged 1.76 per cent, in the darkened tank 1.88 per cent, and again no structural differences were observed in the microscopic structure of the two groups of testes. In these cases some of the animals killed during April presented the white testis and numerous sperm associated with a high degree of activity, and this occurred as early in the darkened tank as in the illuminated one. From these experiments it seems fairly clear that absence of light for at least 4 weeks prior to the breeding season does not inhibit activation of the testis in *Fundulus*. It should, of course, be noted that the animals which developed ripe testes in the dark had been on a rising daylight curve for nearly three months before they were placed in the dark. Whether or not this is a significant factor in initiating the active phase in the testis cycle has not been determined.

Experiments concerning the effect of temperature on activation of the testis gave somewhat different results. Ten animals were kept in a tank in which the temperature of the water averaged 21° C. (variation 19°–21.5°) and 10 animals were kept in a constant temperature room with light conditions similar to those of the first group, the temperature of the water in the aquarium here averaging 5.5° C. (variation 4°–7° C.).

In a series run during December the testis formed as large a percentage of the body weight of animals in the cold room as in the normals (0.53 per cent), but sections of the testes of animals after 23 days in the cold room showed that these were retarded in development, particularly in the later stages of spermatogenesis. In a series run during March and April, moreover, the testes of those maintained at 5.5° C. averaged only 1.16 per cent of the body weight as against 1.76 per cent for those in the warmer room and in general animals maintained in the cold produced sperm much later than did those in the warm room. The retarding effect of the low temperature was noted in this series as early as 9 days after the beginning of the experiment. Only one individual, killed April 5 after 21 days in the lower temperature, showed a degree of activity comparable with that of the control animals.

In brief, then, records have been obtained on 14 animals maintained in a light-proof tank from 4 to 55 days as compared with controls subjected to daylight or to daylight and added night illumination. The testes of these animals were like those of the controls and in cases killed late in April those of both groups were whitish and filled with sperm. Only one case, killed December 26 after 23 days in the dark, showed a testis less developed than that of the control. Records have also been obtained on 17 animals which were maintained at a temperature of 5.5° C. as compared with 13 control animals kept at 21° C. After 9 to 23 days at the lower temperature spermatogenesis was definitely retarded.

These experiments show that the presence of light is not essential for complete activation of the testis of *Fundulus;* and that low temperatures exert a retarding influence on maturation of the sperm. Obviously no evidence is furnished concerning the effects of gradual changes in the amount of light to which the animal is subjected daily, which Hoover and Hubbard found of such importance in the sexual cycle of the trout.

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SOME EXPERIMENTS ON THE RELATION OF THE EXTERNAL ENVIRONMENT TO THE SPERMATOGENETIC CYCLE OF FUNDULUS HETEROCLITUS (L.)[1]

J. WENDELL BURGER

(From Trinity College, Hartford, Connecticut, and The Mt. Desert Island Marine Biological Laboratory, Salsbury Cove, Maine)

INTRODUCTION

Within the last decade a considerable body of experimental work has shown that the sexual cycles of many vertebrates of the north temperate zone are regulated in part by the annual cycle of changes in day-length. Little, however, is known about the relation of the external environment to the sexual cycle of cold water fish. Success in modifying the piscine sexual cycle by light agencies has been reported for the trout (Hoover, 1937; Hoover and Hubbard, 1937), for the minnow, *Phoxinus* (Spaul cited from Rowan, 1938), and for the stickleback (Tinbergen cited from Rowan, 1938). Craig-Bennett (1930) came to the conclusion that the sexual cycle of the stickleback was regulated primarily by temperature. Hoover (private communication to T. H. Bissonnette) has found that light is ineffective on yellow perch which were kept in water below 44° F.

The normal sexual cycle of *Fundulus* has been described by Matthews (1938). As in many cold-blooded vertebrates the sexual cycle is a continuous process throughout, with no genuinely inactive phase, although during the winter there is little or no spermatogenetic activity. In the late summer and fall a limited production of spermatogonia takes place. Vigorous spermiogenesis begins in the spring, with a mating period during May and June. Thereafter occurs a gradual deceleration of spermiogenesis with a concomitant testicular involution.

It is noticed that the major portion of the spermatogenetic activity is present during the spring when the days are increasing in length, and when the temperature of the water is rising. The experiments here reported are to test the relation of light and temperature to the spermatogenetic cycle of *Fundulus*.

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[1] Aided in part by a grant from the American Philosophical Society administered by T. H. Bissonnette for 1938–39.

MATERIALS AND METHODS

Over seven hundred newly captured adult male *Fundulus* were used in four experiments. Two of these experiments were performed in Maine, and two in Connecticut. Fish were secured on June 30 from a tidal inlet on Mt. Desert Island, Maine, and were confined to laboratory aquaria fed by sea water. One control aquarium was placed out-of-doors in a well lighted spot. The fish therein were maintained on natural daylight until August 27. Two other aquaria were placed in a light-proof box, which was illuminated by two 50-watt lamps. From June 30 to July 22 the daily light ration was reduced 20 minutes per day from 15 hours to 8 hours. Then between July 22 and August 27 the daily light ration was increased 20 minutes per day from 8 hours to 20½ hours. The temperature of the aquaria water ranged between 11° and 17° C., in general increasing in warmth from June to August.

On October 29 *Fundulus* were secured from a tidal inlet off Long Island Sound near Niantic, Connecticut. These animals were placed in fresh water aquaria at Hartford, Connecticut. The control fish were exposed to daylight for natural day-lengths between October 29 and January 4. The experimental fish received in addition to natural daylight illumination from a 100-watt lamp. The exposure to electric light was increased every 5 days so that by December 11 the fish were receiving 8½ hours of electric illumination added to normal daylight. After this date no further increases in the length of time of exposure to light were made. The temperature of the fresh water aquaria ranged between 11° and 18° C., decreasing in warmth from October to January.

In a final experiment fish were captured from a tidal inlet at Old Lyme, Connecticut, on February 25, and were confined to fresh water aquaria at Hartford. All aquaria were made light-proof. The fish never received more than 1½ hours of light per day during the experimental period which was from February 25 to March 25. This limited exposure to light was necessary for feeding. One group of males were kept in cold water which varied in temperature between 6° and 10° C. Another group was kept in warmer water which ranged between 14° and 20° C. There was always at least 6° C. difference in temperature between the two groups.

Fish were also sampled from the wild at various intervals. The animals were fed almost daily on either chopped livers or clams. Only healthy, fungus-free fish were killed for histological study.

RESULTS

Confinement to aquaria and to fresh water had no deleterious effects on the testes. Judging from the condition of the internal organs, the diet was more than adequate for the maintenance of good health.

In all the experiments with light, and where there was no significant difference in temperature between controls and experimentals, no difference in the state of the testes was found between control and experimental fish. The experimental light rations were: 21 days of gradually decreased lighting between June 30 and July 22; 37 days of gradually increased lighting between July 22 and August 27; and 68 days of gradually increased lighting between October 29 and January 4.

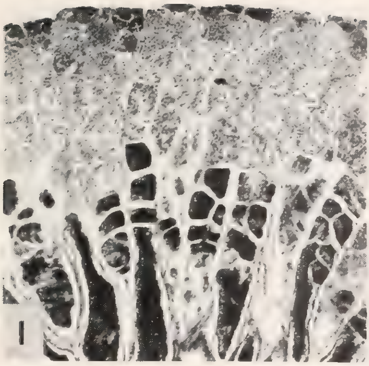
On June 30 at the start of the first experiment, spermiogenesis was at its peak. As can be seen in Fig. 1, there exists at this time a broad zone of cortically located spermatogonia etc. surrounding medullary tubules which are filled with sperm. During the summer there occurs a gradual decrease in the proliferation of spermatogonia, together with a loss of sperm in the tubules. The rate of this testicular involution can be seen by comparing Figs. 1, 2, and 4. Figures 2 and 4 are from laboratory control fish for July 22 and August 27 respectively.

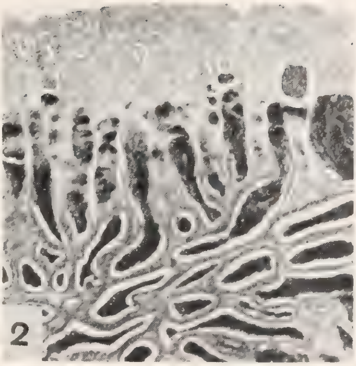
That the fish which received 21 days of gradually decreased lighting showed no differences in testicular state when compared with control fish, is illustrated in Figs. 2 and 3. Figure 2 is from a control testis, and Fig. 3 from an experimental fish whose light ration was gradually reduced to 8 hours per day. Likewise a comparison of Figs. 4 and 5 shows that there was no difference in testicular state between control fish (Fig. 4) and experimental fish (Fig. 5) after 37 days of gradually increased lighting applied to *Fundulus* which previously experienced 21 days of decreased lighting.

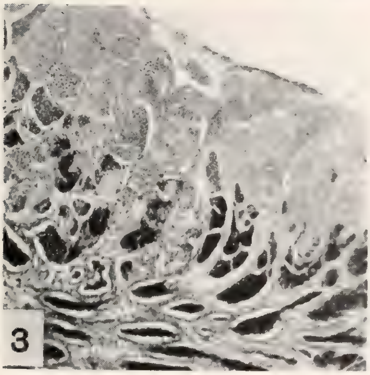
These results indicate that the testicular involution which normally occurs during the summer when the day-lengths are naturally shortened cannot be hastened by 21 days of decreased lighting. Moreover, 37 days of subsequent increased lighting does not change the rate of testicular involution, nor does it induce a precocious new spermatogenetic cycle. Since many animals are refractory to photoperiodic manipulations at the end of their sexual cycle, the above experiments are no test for the efficacy of photoperiodic manipulations on the sexual cycle.

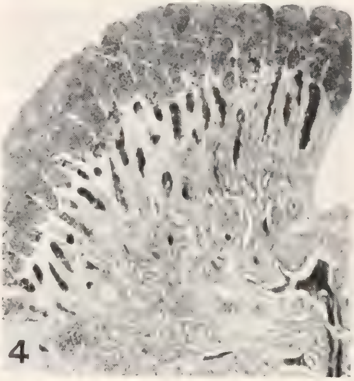
The fish which were lighted for 68 days between October 29 and January 4 offer a fair test, however, as to whether or not the sexual cycle of *Fundulus* can be influenced by light. When this experiment was begun, the cortical zone of the testis was slowly proliferating spermatogonia, while the medullary system of tubules was involuted and devoid

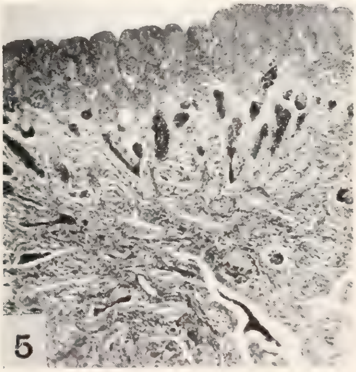
PLATE I











All figures are unretouched photomicrographs, × 80.

FIG. 1. Section of a testis from a fish captured 6/30/38. The cortical zone (at the top of the figure) of spermatogonia, etc. is broad; the medullary zone of tubules is black with sperm.

FIG. 2. Section of a testis from a laboratory control, 7/22/38. The spermatogonia are fewer than in Fig. 1, and the tubules contain fewer sperms.

FIG. 3. Section of a testis, 7/22/38 after 21 days of shortened day-lengths. The testis is in the same condition as that of the controls (Fig. 2).

FIG. 4. Section of a testis from a laboratory control, 8/27/38. Spermiogenesis is almost finished; the tubules have markedly involuted.

FIG. 5. Section of a testis, 8/27/38 after 37 days of increased lighting. No significant difference is found between this and the control (Fig. 4).

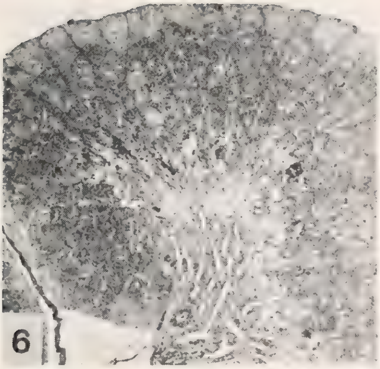
of sperm. This condition can be seen in Fig. 6, which is a section of a testis on October 29.

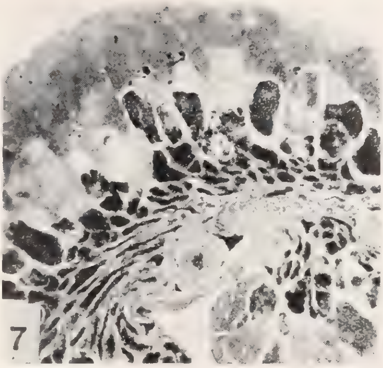
During this experiment sperm were produced both by the control and experimental fish. These sperm can be seen in Fig. 7 which is from a control fish on January 4, and in Fig. 8 which is from an experimentally lighted fish on January 4. In the control fish, sperm were formed while the days were decreasing in length, as indicated by fish sampled in December. In the experimental fish, sperm were formed no more abundantly when the day-lengths were increased in length by means of 8½ hours of electric light added at night. Thus for fish at the threshold of a new spermatogenetic cycle, the application of increased or decreased daily rations of light does not modify the rate of the subsequent formation of sperm.

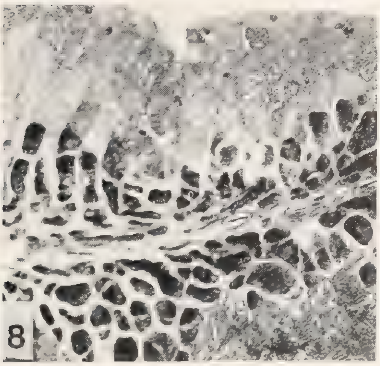
A comparison of laboratory fish and fish from nature in early January showed that the fish in their natural habitat do not form sperm at this time as did the laboratory fish. This statement needs to be qualified slightly for there are *Fundulus* in nature which during the winter form a very few spermatozoa. However, the general condition for winter fish at least up until early March is similar to that shown in Fig. 6. The most obvious difference between the laboratory fish and the fish from nature is the difference in water temperatures. The laboratory fish lived in water between 11° and 18° C., while the fish in nature during the winter lived in water whose temperature was near 0° C.

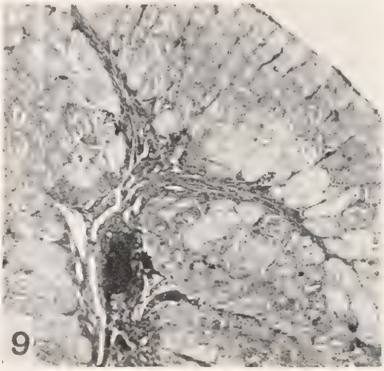
The experiment with temperature where the daily light ration was only long enough for feeding the fish indicates that spermatogenesis is responsive to temperature manipulations. Figure 9 is a section from a testis from a fish after 29 days in water whose temperature varied between 6° and 10° C. These temperatures were somewhat higher than those experienced by the fish in nature at the time of capture and during the experimental period. This testis is a winter testis and shows no transformations of spermatozoa. However, there did occur a slight multiplication of spermatogonia so that the testis was not completely inactive during this period. Figure 10 is a section of a testis from a fish after the same 29 days in water whose temperature varied between 14° and 20° C. Here spermatozoa have been formed in large numbers. This effect of higher temperature can readily be seen by comparing Figs. 9 and 10. This result was uniform for all fish. It should be emphasized that these fish never had more than 1½ hours of light per day during the experimental period, and usually not more than one-half hour.

PLATE II









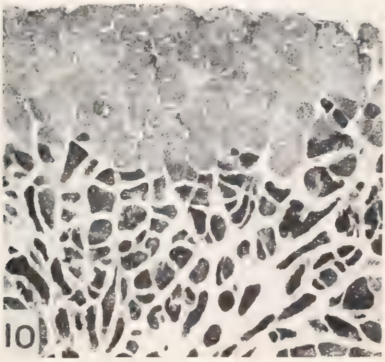


FIG. 6. Section of a testis from a fish captured 10/29/38. The bulk of the testis consists of spermatogonia. Sperms are absent and the tubular system is greatly reduced.

FIG. 7. A section from a laboratory control, 1/4/39. The black areas are tubules which contain sperm.

FIG. 8. A section of a testis, 1/4/39 after 68 days of increased lighting. Sperms while abundant are no more numerous than in the controls (Fig. 7).

FIG. 9. A section of a testis, 3/25/39 after 29 days in almost complete darkness in water whose temperature varied between 6° and 10° C. This is essentially a winter testis consisting of spermatogonia (compare with Fig. 6). Sperms are absent.

FIG. 10. A section of a testis, 3/25/39 after 29 days in almost complete darkness in water whose temperature varied between 14° and 20° C. The black areas show the large numbers of sperms that have formed.

DISCUSSION

These experiments indicate that light as such is of no importance in the spermatogenesis of *Fundulus*. Spermatozoa can be formed in almost complete darkness, and will form in equal abundance when the days are either increasing or decreasing in length. Temperature appears as the important factor of the external environment which modifies spermatogenesis. In cold water spermatogenesis is retarded or inhibited, while in warm water spermatogenesis is rapidly completed.

These experiments give no exact data on the critical temperatures involved, but from our observations both on experimental fish and on fish in nature a general scheme seems clear. At temperatures near 0° C. the testis is inactivated. As the temperature rises toward or around 10° C. spermatogonial multiplications occur. Still higher temperatures permit the transformations of sperm to take place.

Marine temperatures show a very orderly annual cycle. Dr. R. A. Goffin kindly gave us the mean daily sea water temperatures for 1938 at Woods Hole, Massachusetts. Dr. V. L. Loosanoff also referred us to his paper (Loosanoff, 1937) which gives the shallow water temperatures for over three years at Charles Island, Long Island Sound. Both sets of data show a low point in February followed by a continued rise beginning in March and reaching a maximum in August. From August to September a continued drop takes place.

The spermatogenetic cycle of *Fundulus* fits nicely into this annual temperature curve. In the fall as the temperature drops spermatogonial multiplications take place. During the winter the testis is relatively inactive. In fact, fish captured in late February show slightly less testicular activity than those captured in early January. The spring rise in water temperature is accompanied by increased spermatogenetic activity. It should be remembered that in nature the beginning of active spermatogenesis coincides with the warming of the water, and not with the increased lengthening of the days which began three months previously.

SUMMARY AND CONCLUSIONS

1. No differences in the velocity of the spermatogenetic cycle of adult male *Fundulus* were found between control and experimental fish kept in water of the same temperature when treated as follows: (*a*) 21 days of gradually decreased day-lengths between June 30 and July 22, (*b*) 37 days of gradually increased lighting subsequent to treatment as in (*a*) between July 22 and August 27, (*c*) 68 days of increased lighting between October 29 and January 4.

2. Laboratory fish kept during the late fall and early winter in

water whose temperature was higher than that experienced by fish in nature showed an acceleration of spermatogenesis.

3. Laboratory fish which received no more than 1½ hours of light per day and which were kept in water of from 6° to 10° C. between February 25 and March 25 remained inactive sexually. Fish which received no more than 1½ hours of light per day and which were kept in water whose temperature varied between 14° and 20° C. formed large numbers of sperm within this same period of time.

4. It is concluded: (*a*) that the spermatogenetic stages of the annual sexual cycle are not affected by light as light; (*b*) that the temperature of the water is the important factor of the external environment regulating spermatogenesis in *Fundulus*.

5. It is suggested that at temperatures near 0° C. sexual activity is inhibited. As the temperature rises toward or near 10° C. spermatogonial multiplications occur. Still higher temperatures produce complete spermatogenesis.

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INFLUENCE OF THE SINUSGLAND OF CRUSTACEANS ON NORMAL VIABILITY AND ECDYSIS[1]

F. A. BROWN, JR., AND ONA CUNNINGHAM

(*From the Zoölogical Laboratory, Northwestern University*)

Since the work of Perkins and of Koller in 1928, who independently described the presence of a substance in eyestalk extract which exercises a very potent effect upon the chromatophores of crustaceans, there has been much interest shown in the crustacean eyestalk function. The picture has been rendered even more interesting as a result of the work of Brown (1935) and of Kleinholz (1938), demonstrating that humoral activity in this group of animals is by no means a simple one, but that several hormonal substances are normally functioning. Hanström (1935) performed experiments in which he showed that the portion of the eyestalk which was active in affecting chromatophores always contained, among other things, a tissue which he has termed the sinusgland. This has given rather good evidence indicating which tissue of the eyestalk is the active one in this regard. The cells of this tissue were shown to be secretory in nature and to contain a rich supply of secretory granules. The more recent work of Hanström (1936), Stahl (1938) and others have shown the sinusgland to be present in some degree in all the crustaceans that have been examined in detail. Its occurrence appears to be quite independent of the state of development of a chromatophore system. Functionally it appears to have common properties with the corpora allata of insects since an extract of the latter organ in many cases serves as an activator of crustacean chromatophores. Abramowitz (1936, 1938) has demonstrated that the chromatophorotropic substance from the sinusgland and the intermedin of the vertebrates have certain common chemical and physiological properties.

Koller (1930) was the first to demonstrate that the eyestalk substance has another function in addition to the control of chromatophores. He found that animals from which the eyestalks had been removed failed to deposit calcium in their exoskeletons to the same extent as normal animals. He interpreted this to be the result of removal of the source of a controlling hormone. Welsh (1937) found that when he perfused an exposed crayfish heart with eyestalk extract

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[1] This investigation was supported by a research grant from the Graduate School of Northwestern University.

there was a pronounced speeding up of that organ. Brown (1938) demonstrated that removal of the eyestalks appreciably shortened the life of the individual and that the shortening thus induced could be compensated for in part by implantation of eyestalk tissue into the ventral abdomen. This shortening of the life of the animal has been called a "viability effect" of an eyestalk hormone, though it is fully realized that this is a function described in far too general terms. It is hoped that this "viability effect" can soon be analyzed into the particular phenomena responsible for the shorter life.

There has frequently been suggestion of a "molting effect" of the eyestalk substances, though no adequate data have yet been published to establish such a function. The only grounds for such a belief are that several investigators have mentioned that eyestalkless animals appear to molt more frequently than normal ones. No reason has been advanced for thinking the effect is due to anything other than the injury caused by the operation of eyestalk removal (indicated by Darby, 1938).

The following research has been conducted in continuation of that of Brown (1938) with the intention of discovering just what tissue of the eyestalk is responsible for the "viability effect" of this organ. There is included here the first direct evidence for an endocrine activity of the sinusgland of the crustacean. Hitherto its functioning had been supposed upon the grounds of the best of circumstantial evidence. During these experiments the sinusgland has been dissected out and implanted into the ventral abdominal sinus of eyestalkless animals. Direct physiological evidence of its endocrine function has been demonstrated. Furthermore, it is quite well established as a result of these experiments that this gland is the one responsible for the normal continuation of life of the animal and also that it has a functional activity in the control of molting. The possibility of explaining the viability effect of eyestalk hormones in terms of molt control will be discussed.

Methods and Materials

All the crayfishes used in these experiments were small individuals (carapace lengths 15–30 mm.) of the species *Cambarus immunis*, with the exception of certain large individuals (*Cambarus virilis*, *C. blandingii*, and *C. immunis* of carapace lengths 30–40 mm.) which were used as the source of the sinusgland for implantation. The animals were brought into the laboratory a few days before the beginning of an experiment. It was our purpose to use experimental extirpation and implantation to determine the normal functions of the eyestalk gland within the body.

The method of extirpation was simple: the eyestalks were removed as a whole and the wound sealed with an electric cautery. By so sealing the wound, less than 10 per cent of the animals died as a result of the operation. It is fully realized that such a method of gland extirpation removed much tissue in addition to that of the sinusgland.

In the first experiment to be described the implantation consisted of all the eyestalk tissue. The eyestalks were removed from an animal and dropped into amphibian Ringer's solution. With the aid of a dissecting microscope the exoskeleton of the eye was cut away. The soft parts of the eyestalk were easily removed with fine forceps. This tissue was then teased into minute fragments and injected by means of a glass capillary pipette into the ventral sinus of the abdomen. The glass pipette proved to be especially satisfactory since it was possible to ascertain that all of the tissue entered the animal and none was left adhering to the walls of the pipette.

In those experiments in which the sinusgland by itself was to be implanted the gland was carefully dissected out in the following manner: the eyestalk was removed from a large crayfish and dropped into a watchglass containing amphibian Ringer's solution or a balanced salt solution based on Griffeths' analysis of *Astacus* blood (which will henceforth be referred to as Griffeths' solution). With a pair of sharp pointed scissors the chitinous exoskeleton was clipped to free the dorsal half of the stalk skeleton from the ventral half. The contents of the stalk were then picked out with fine pointed watch-maker's forceps and the dorsal tissue was teased away in the direct light of a strong lamp. The sinusgland tissue stood out quite conspicuously as a seemingly fibrous and granular bluish tissue. This mass of tissue was easily torn away from the adjacent nerve tissue. All the adhering tissue was teased away and the gland rinsed in amphibian Ringer's or Griffeths' solution. With forceps the gland was next pushed through an opening made in the ventral side of the abdomen. The clear exoskeleton in this region made it possible to ascertain that the minute gland was actually left in place upon removal of the forceps.

In order to determine the exact location of the tissue removed from the eyestalk, sections were made of the bluish gland-like tissue that was removed, and also of all the remaining portions of the eyestalk. In addition, longitudinal sagittal sections of the complete eyestalk were made as a control. By study of these three sets of sections it was readily determined just what tissue was being implanted. It was discovered that the implant tissue in histological section appeared to be definitely glandular in nature and occupied a position wedged between the medulla externa and the medulla interna. Considering its position and the fact that its cytoplasm was richly charged with eosinophilic inclusions, it seemed highly probable that this gland was the same as that described by Hanström (1936) as the sinusgland. The accompanying photographs show this gland as it occurs in *Cambarus virilis*. The first photograph is a median sagittal section of the

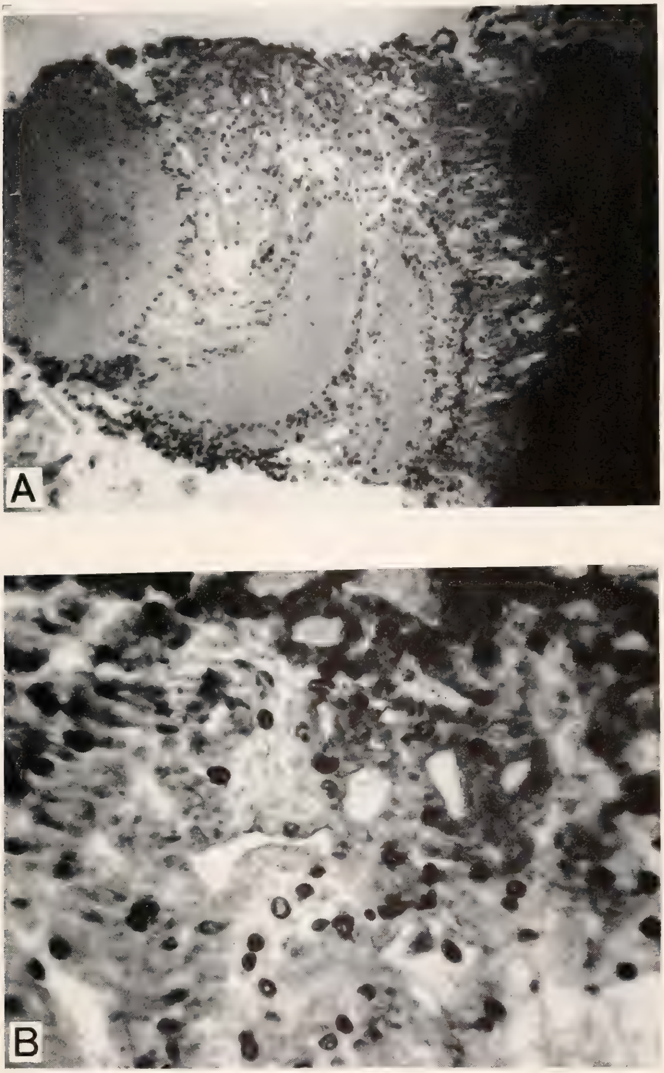

FIG. 1. Sagittal sections through the eyestalk of *Cambarus virilis* (6 micra thick and stained with Delafield's haematoxylin and eosin). *A*. At a magnification of 80 ×, showing the sinusgland as a somewhat triangular section of tissue located dorsally to a point intermediate between the medulla externa and the medulla interna. *B*. A higher magnification (360 ×) of the central region of the sinusgland.

eyestalk at a magnification of approximately 80× and the second is a higher power magnification (about 360×) in the central region of the gland.

During the experimental period all the animals were kept in individual glass finger bowls in water not quite deep enough to cover the carapace. These finger bowls were covered loosely with glass plates to minimize evaporation of the water but still to permit circulation of air over the water surface.

The experiments performed included extirpation and implantation, with appropriate controls, and observations were made upon viability and molt behavior.

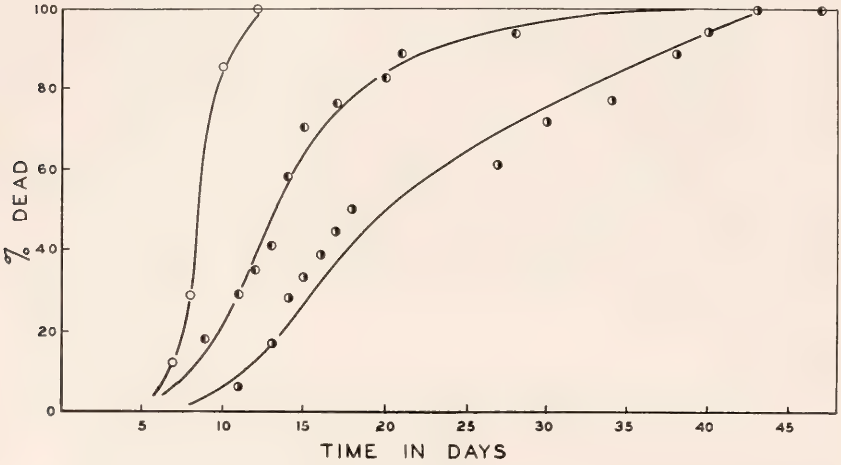

FIG. 2. The relation between the percentage of animals dead and the number of post-operative days for eyestalkless crayfishes, (○); eyestalkless crayfishes with a heteroplastic implant of sinusglandless eyestalk tissue, (◐); and eyestalkless crayfishes with only a heteroplastically implanted sinusgland, (◑).

EXPERIMENTS ON VIABILITY EFFECTS

Experiment I

The animals of this experiment, all *Cambarus immunis* with both eyestalks removed and the stubs cauterized, were divided into three lots. In the first lot were 7 animals with no further treatment. The second lot of 17 animals had a sinusgland taken from a single eyestalk of a large *Cambarus virilis* or *Cambarus blandingii acutus* implanted into the ventral sinus of their abdomens. The third lot of 18 animals had an abdominal implantation consisting of all the eyestalk tissue of a single eyestalk of *Cambarus virilis* or *Cambarus blandingii acutus*, from which the gland had been carefully removed.

The results of this experiment are best shown in the form of a graph (Fig. 2) in which the percentage of animals dead is plotted against the post-operational day. This graph demonstrates clearly that eyestalkless animals without abdominal implants live significantly shorter lengths of time than eyestalkless animals into which eyestalk tissue *minus the sinusgland* has been implanted. Similarly, eyestalkless animals which have received abdominal implants of the minute sinusgland by itself, live very significantly longer than those animals into which the remaining portion of the eyestalk tissue was implanted. Comparing only the instance of sinusgland implant with the case of no implant, we can conclude definitely that the minute sinusgland lengthens the post-operative life of the animal considerably. It is well to bear in mind that these two latter groups have been subjected to operations of different degrees of severity, in which the animals which live longer have been subjected to more severe operative injury, the animals of the latter group having their abdomens punctured as well as having both eyes removed. A logical explanation of the intermediate length of post-operative life in the instance of those animals with the glandless stalk tissue implants is that there is present in the blood spaces of the general eyestalk tissue a product that has arisen from the sinusgland. During its removal, a bluish liquid is seen to diffuse out of the gland and infiltrate into the surrounding tissues. The general stalk tissue is frequently filled with a homogeneous blue liquid which, in all probability, comes from the same origin. We believe, therefore, that this additional substance is responsible for permitting these animals to live longer than those in which no implant

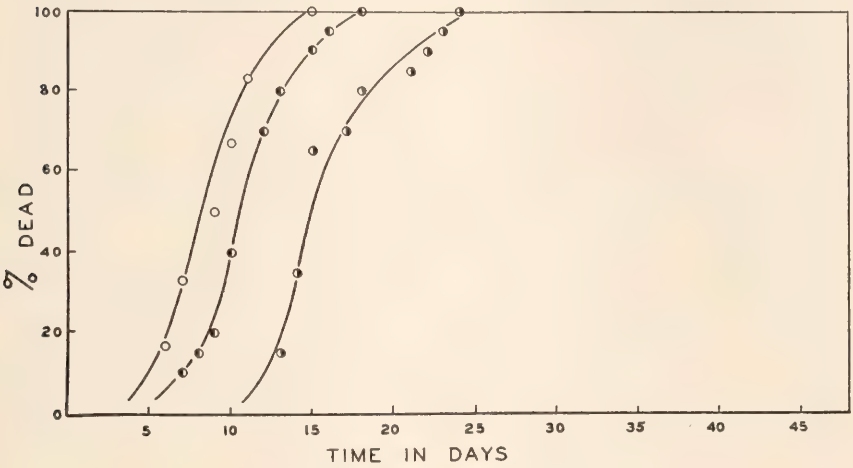

Fig. 3. The relation between the percentage of animals dead and the number of post-operative days for eyestalkless crayfishes, (○); eyestalkless crayfishes with a homoplastic implant of sinusglandless eyestalk tissue, (◐); and eyestalkless crayfishes with only a homoplastically implanted sinusgland, (◑).

is made. It is also possible that fragments of the gland itself still remain which were not removed at the time of operation.

The implants in this experiment are heteroplastic, while in an experiment to be described later all the implantations were autoplastic as was the case with those observations published by Brown (1938). It becomes doubly interesting that the sinusgland has a definite effect not only upon the length of post-operative life in the same species of animal, but that the tissue from one species is capable of working effectively within the body of another species to the same end. Thus these substances, or this substance, is inter-specifically active.

Experiment II

In this experiment, like the preceding one, eyestalkless *Cambarus immunis* were divided into three lots. In the first lot, consisting of 6 large animals, there was no further treatment. A single sinusgland from an eyestalk of a large animal of the same species was abdominally implanted into each of the 20 small animals of the second lot. Each of the 20 animals of the third group received an abdominal implant consisting of the tissue from a single large eyestalk from which the gland had been removed.

The results of this experiment are shown in Fig. 2.

This experiment confirms the influence of the sinusgland on viability demonstrated in Experiment I. Here the implantations were homoplastic, from large *Cambarus immunis* to small *Cambarus immunis*. As in Experiment I, the animals without any implant lived a much shorter time than those with sinusgland implants, and animals in which sinusglandless eyestalk tissue was implanted lived for an intermediate length of time.

EXPERIMENTS ON THE MOLTING CONTROL FUNCTION OF THE SINUSGLAND

Experiment I

This experiment was intended to discover any differences that might occur in the molting process among animals from which both sinusglands had been removed, one sinusgland removed, both sinusglands removed but with them autoplastically implanted into the ventral abdominal sinus, and finally, completely normal animals.

In this experiment four lots of animals were isolated. The first lot of 34 animals was left in perfectly normal condition, though placed in the usual individual glass finger bowls with covers. The second lot of 48 animals was subjected to removal of one eye each. A third lot of 79 animals had both eyestalks removed in the usual manner. The fourth lot of 44 animals had both eyestalks removed and the contents of their own eyestalks in amphibian Ringer's solution injected into the ventral sinus of the abdomen. Observations were made only with regard to actual molting. The results that were obtained are summarized in Table I.

TABLE I

Data indicating the extent of molting in crayfishes under different experimental conditions.

	Normal Animals	One Eye Off	Two Eyes Off	Two Eyes Off (Implant)
Total no. examined....	34	48	79	44
No. "molts".........	9	19	23	3
Per cent "molts".....	26	40	29	7
Per cent "molts" dying in process..........	44	16	74	100
Per cent molt/av. life span...............	2.0	3.4	5.75	1.0

All the records of molting in Table I indicate instances in which the animal either completed the molt or was well along in the process at the time of death. The most significant portion of the table is the item "per cent molt/average life span" which gives the only true figure of the relative rates of molt. The "per cent molts" fail to do this inasmuch as the different lots of animals survived different lengths of time; consequently such animals as normal animals and those with one eyestalk off had a longer time in which molts could occur. On this strictly relative behavior (per cent molt/average life span) the figure for normal animals is 2. With one eye removed, the rate of molt is increased by about 75 per cent, and with the removal of two eyes the molting has been accelerated about 200 per cent. The striking fact, however, is that when both eyes were removed and the eyestalk tissue abdominally implanted, the figure indicating the molting rate is 1, or about half that of normal animals. Were it not for the anomalous molting rate of this last group the results could be interpreted as indicating that the rate of molting is a function of the extent of injury. But, taking the data together, there appears to be a more probable explanation. The eyestalk tissue, under nerve control, liberates a humoral substance into the blood which inhibits the molt. With one eye removed, relatively less substance is liberated and with two eyes removed none of the material, and we see molt correspondingly going on at relatively greater rates. In these terms the explanation of behavior of the last group of animals might be that the implanted glandular tissue continuously liberates some antimolting substance and the animal is almost unable to molt.

Some of the acceleration resulting after eyestalk removal may be due to injury effects, but that they are not totally due to injury is indicated by the implantation experiments.

Experiment II

This experiment points to the sinusgland in the eyestalk as the actual tissue involved in the formation of the molt control humoral substance. The data for this conclusion are taken from observations on molting in the animals in Experiment II on viability.

A consideration of the ratio of percentage of completed or nearly completed molts to average survival period, shows that the implantation of the sinusgland reduces the molting rate to about one-fifth of that which occurs in the controls with the glandless stalk tissue implants. The conclusions of the former experiment are confirmed and it is further indicated that the sinusgland is the effective tissue in molt control. The results of this experiment are summarized in Table II.

TABLE II

Data indicating the extent of molting in crayfishes under different experimental conditions.

	Two Eyes Off	Two Eyes Off (Implant)
Total no. examined	20	20
No. "molts"	4	1
Per cent molts	20	5
Per cent molts/av. life span	1.57	.31

In the course of this experiment all the animals were carefully watched, not only for completed molts but also for the slightest symptoms of the beginnings of molt. The early signs of molt were usually indicated by a visible separation between the carapace and the first abdominal tergite. Practically all of the eyestalkless animals, regardless of the type of implant, showed this separation from three hours to three or four days prior to their death. This was so definite that it was possible to predict the death of any animal within these limits. In many instances this separation was followed by a completed molt, though in the majority of cases the animals died before further steps in the molting process. It is admitted that some other factors, such as change in general tone of the abdominal musculature or upset in the water metabolism of the animal, might be operating in inducing the separation of these two skeletal elements. Superficially, however, we are unable to differentiate between the initiation of the normal molt and its induction by other causes. Furthermore, many of the animals showing this apparent initiation in the molt process showed muscular activity of the body such as is usually associated with the normal molting process.

Those animals from which the eyestalks had been removed and which received the glandless eyestalk implantation, all showed the apparent initiation of molt or completed the molt prior to their death. In three cases the animals completed the molt before death, in one case dying within a day of the molt and in the other cases living two and four days, respectively, after molting. In a fourth case the animal died when well along in the molting process. These facts would indicate that even without the eyestalks the animals are physiologically able to complete the molt. But the fact that the eyestalkless animals sometimes continue to live several days after molting and then die without showing further signs of molt, indicates that the sinusgland has a function in addition to molt control.

The majority of the animals with sinusgland implants also showed the beginnings of molting prior to their death, just as did the first lot. The only difference between the lots seemed to be that the molting activity was postponed in the case of the implanted animals. These animals seldom do more than show this first sign of molt, scarcely ever proceeding far into the molt or completing it. A possible explanation of this is that these animals are prevented from molting by action of the implant until the absence of the eyestalk has worked other degenerating effects upon the organisms to the extent that they no longer have the power to go far with the molt, in spite of removal of the inhibitor through loss of function of the implant. In this regard it would be interesting to trace the rate of degeneration of the implanted tissue to see if there may be any correspondence between the time of oncome of the molt and the structural degeneration of the implanted cells.

It may be possible to interpret the data of Koller (1930) in terms of molt control activity. Animals molting more frequently as a result of absence of a hormone from the sinusgland might well be expected to have less calcium salts in their exoskeleton than normally.

SUMMARY

1. Direct evidence for an endocrine activity of the crustacean sinusgland is given. This evidence has originated from implantation experiments.

2. Removal of the sinusgland significantly shortens the life of the animals, and conversely the length of life of animals with sinusglands removed can be significantly lengthened by implantation of the gland.

3. The sinusgland is readily dissected out in fresh eyestalk tissue

in strong reflected light. It has a distinctly bluish cast. It is a definite organ which can be readily teased away from the surrounding tissue and removed as a whole.

4. Certain evidence suggests very strongly that a substance concerned with the control of molting is elaborated in this gland. The most probable action of this substance is that of inhibiting molt.

5. The action of the sinusgland in molt control appears to be insufficient to explain the viability effect entirely.

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THE METHOD OF FEEDING OF CHAETOPTERUS

G. E. MACGINITIE

(From the William G. Kerckhoff Marine Laboratory of the California Institute of Technology, Corona del Mar, California)

INTRODUCTION

Ciliated currents present on the surface of animals, when examined under artificial conditions, are seldom, if ever, typical of the animal in its natural environment. Failure to recognize this fact and failure to observe the presence of mucus and note its importance in the feeding process have given rise to many erroneous descriptions of the feeding mechanism of various marine invertebrates. In conformity with the statement made in *Science* (MacGinitie, 1937), the feeding activities of many marine invertebrates have been investigated (including tunicates, pelecypods, gastropods, annelids and coelenterates), and descriptions of the feeding activities of these animals will follow as soon as they can be prepared for publication. This paper will deal with the feeding of the annelid *Chaetopterus variopedatus* Rénier et Claparède.

Because of its wide distribution and its usefulness as a source of embryological material, *Chaetopterus* is well known both abroad and in this country. Also, because of its unusual and somewhat bizarre structure, it has created a great deal of interest from both an anatomical and a natural history point of view (Laffuie, 1890; Enders, 1909). However, no paper that I have seen has given the correct method of feeding of this animal.

FEEDING METHOD

The structures concerned with the feeding activities of *Chaetopterus* are the peristomial funnel with its lips, the mouth, the dorsal ciliated groove, which ends in the dorsal cupule of the thirteenth segment, the pair of aliform notopodia of the twelfth segment, and the three fans of the fourteenth, fifteenth and sixteenth segments (see Fig. 1).

In preparing to feed, *Chaetopterus* approaches one or the other end of the leathery U-shaped tube in which it lives and spreads its aliform notopodia out against the sides of the tube. It then begins to secrete mucus from the inner walls of these notopodia, the secretion beginning at the distal ends and proceeding inward toward the body. The cilia of the inner surface of the notopodia carry the mucus across the opening in a sheet from the distal ends to the body of the worm, whence it is carried posteriorly as a bag by the ciliated groove to the dorsal cupule, where the closed end of the mucous bag is taken into the cup or concave surface of this organ. This creates an elongated bag of mucus, the anterior end of which is fastened to or continuous with the glands lining the inner surface of the aliform notopodia, and the closed posterior end of which is held by and rolled up within the dorsal cupule.

A current of water is now maintained through the burrow by the activity of the three fans just posterior to the dorsal cupule. Since the walls of the burrow are completely in contact with the body of the animal and the aliform notopodia at the anterior end of the mucous bag, it is necessary for the current of water to pass into the bag, out through its sides, and thence along the body of the worm, and ultimately to issue from the burrow at the opposite end. While the current is being maintained by the fans, mucus is continuously secreted at the anterior end of the bag, and, at the same rate, the posterior end is rolled into a ball within the dorsal cupule by the cilia of its inner surface. Since all water entering the burrow while a mucous bag is

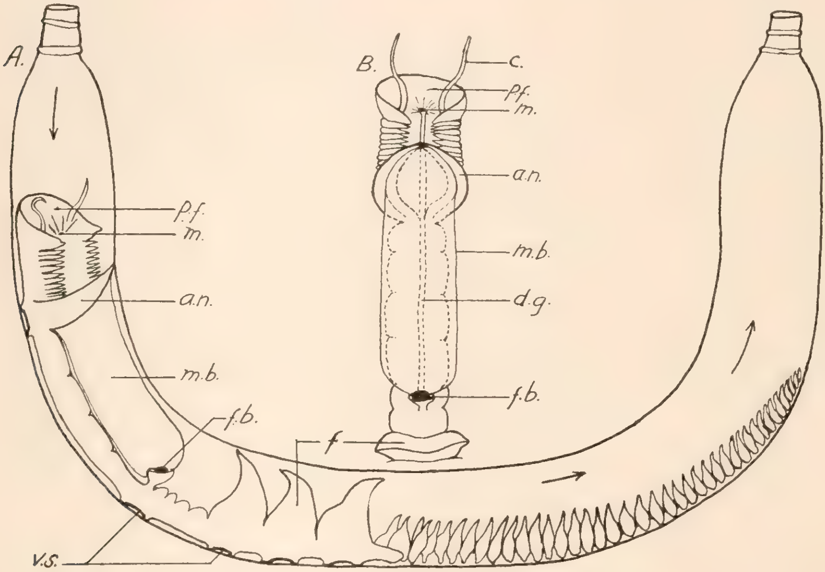

FIG. 1. *A*, *Chaetopterus variopedatus* within its tube, feeding; *B*, dorsal surface of anterior portion of worm. *a.n.*, aliform notopodium; *c.*, cirrus; *f.*, fans; *f.b.*, food ball being rolled up within the dorsal cupule; *d.g.*, dorsal ciliated groove; *m.*, mouth; *m.b.*, mucous bag; *p.f.*, peristomial funnel; *v.s.*, ventral suckers.

present passes through the walls of the bag, the mucus removes from the current all solid particles, whatever their size. It is these particles which lodge on the inner surface of the mucous bag that constitute the food of *Chaetopterus*. It consists mainly of detritus (organic debris and bacteria) stirred up from the surface of the ocean or estuarine bottom by wave action, currents, other animals, etc.

Because the entrances to the tube of *Chaetopterus* are considerably constricted, no very large particles find their way in with the feeding current. Such that do are usually detected by the peristomial cilia of the worm and are passed out at the sides of the worm anterior to the aliform notopodia, which are lifted to allow the material to pass, and so do not find lodgment in the mucous bag. Since the mucus of the bag is being secreted continuously, and at the same time the posterior end is being rolled into a ball in the dorsal cupule, it is evident that the entire bag is constantly being renewed, and that the posterior portion is much more heavily laden with food than is the anterior.

When the ball of mucus and food in the dorsal cupule reaches a certain size, the anterior end of the mucous bag is cut off from the notopodia, and the dorsal cupule continues to rotate the ball until the remainder of the bag is completely (or, occasionally, only partly) rolled up. The dorsal cupule is then turned anteriorly and stretched forward somewhat to expel the ball of food onto the posterior end of the dorsal groove. At the same time the action of the cilia of the groove is reversed, and the bolus of mucus with its entrapped food is carried forward along it to the mouth, where the bolus is enveloped by the lips and swallowed.

The size of the bolus of food depends upon the size of the dorsal cupule, and, therefore, upon the size of the animal. For a *Chaetopterus* about 6 inches long the food ball averages about 3 mm. in diameter. When *Chaetopterus* is feeding there is some variation in the length of its body, particularly in that portion between the head and the dorsal cupule, and, therefore, the length of the mucous bag will vary in the same animal at different times.

The following figures are given for a worm 142 mm. in length, measured during a time when the animal was feeding. Fifteen millimeters posterior to its point of origin, the width of the mucous bag was 6 mm., and the dorso-ventral diameter at the same point was 7 mm. The length of the mucous bag was 37 mm. The rate of secretion of this bag was approximately 1 mm. per second. While the worm was feeding the number of beats for any one of the three fans was 64 per minute, and this rate was the same for this particular worm as observed on successive days over a period of several weeks. Although the rate of beating of the fans is quite uniform for any one worm, it varies with individuals, for another worm maintained a rate of 52 beats per minute. From the beginning of the spinning of the mucous bag to the ingestion of the bolus of food required, on the average, 17 minutes, and varied only plus or minus 1 minute from this average.

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THE ACTION OF EYE-STALK EXTRACTS ON RETINAL PIGMENT MIGRATION IN THE CRAYFISH, CAMBARUS BARTONI

JOHN H. WELSH

(*From the Biological Laboratories, Harvard University*)

I

Pigment cells of the retina of the vertebrate eye and pigment cells of the compound eye of arthropods have long been known to lack motor innervation. Hence there has been much speculation regarding the nature of the mechanisms controlling the movements of these cells or the pigment within them. As recently as 1932 when Parker reviewed the literature on retinal pigments there was no direct evidence as to the nature of the control, but considerable indirect evidence suggested that hormonal agents were responsible for initiating and maintaining retinal pigment migration. The first successful attempt to demonstrate the existence of a hormone acting on the retinal pigments of arthropods was made by Kleinholz (1934; 1936). He found that the injection of an active principle from eye-stalks of *Palaemonetes* into dark-adapted individuals of the same species caused the movement of the distal and reflecting pigments to positions characteristic of the light.

Studies of the persistence, under constant external conditions, of 24-hour cycles of pigment migration in the compound eye had led to one of the earlier suggestions that there was a hormonal control of retinal pigment (Welsh, 1930; see also Welsh, 1938, for review of the literature pertaining to diurnal rhythms). The extension of these studies to the eye of *Cambarus* made necessary an investigation of hormone factors in the control of retinal pigment migration in this crustacean. Certain of the results obtained will be presented in this paper.

II

The majority of observations were made on eyes of *Cambarus bartoni* but eye-stalks of *C. clarkii* and *C. limosus* were sometimes used as sources of the pigment-activating substance.

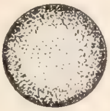
The approximate positions of the three sets of pigment (distal, proximal and reflecting) were determined by briefly illuminating the eye of an animal, in the dark, by a bright beam of light, and observing the amount of light reflected from the eye. This method has been employed by Day (1911). Exact determinations of pigment positions were first made by sectioning the eyes but this is a time-consuming procedure and a rapid method was developed as follows. Animals were killed by dipping in water at 80° C. for 10–15 seconds. The eyes were then removed and split in halves. When these halves were examined under a binocular, using bright reflected light, it was possible to measure the positions of the pigments quite as accurately as in sections.

The active substance from the eye-stalk, which may be identical with the chromatophorotropic hormone (Abramowitz and Abramowitz, 1938), was prepared by grinding 20 eyes and eye-stalks of medium-sized crayfishes in 1 cc. of cold-blooded Ringer, then heating to 100° C. and filtering. The injection of an appropriate volume of the filtrate made it possible to administer the active material from a fraction of an eye-stalk or from one or more eye-stalks as a given dose. Appropriate control injections of Ringer's fluid and of extracts of ventral nerve cord were made and always with negative results.

III

The first experiments to be reported were done to test the effect of the eye-stalk extract on light-adapted eyes. Several *C. bartoni* were allowed to adapt for several hours in bright diffuse sunlight. At 10:30 A.M. 0.05 cc. of eye-stalk extract (containing the active material from one eye-stalk) was injected in the ventral abdominal musculature of each of half the individuals. At 2:00 P.M. the entire lot was killed with hot water and the eyes removed, split and examined.

In Fig. 1 may be seen the distribution of the pigments of a light-adapted eye. The distal pigment forms a sheath around the cone and the process leading from the cone to the rhabdome, but a portion of each distal pigment cell next to the retinular or proximal pigment cells is not filled with pigment. Most of the proximal pigment surrounds the rhabdome, but some remains below the basement membrane. The reflecting pigment in crayfish eyes does not migrate as it does in some crustaceans (Welsh, 1932). The appearance of an eye of a light-adapted animal, when viewed by reflected light, is shown in Fig. 1*a*. The positions of the black screening pigments are such that light cannot reach the reflecting pigment layer nor can light rays, except those which are parallel to the main axis of an ommatidium, reach the rhabdome or light-sensitive element of the eye. Such an eye is called an apposition eye since a given rhabdome receives light only from its adjacent lens system and is not acted on by light entering at an angle through neighboring ommatidia.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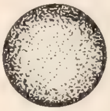
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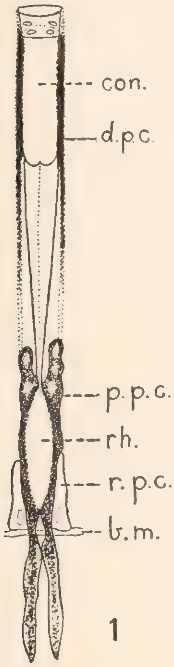

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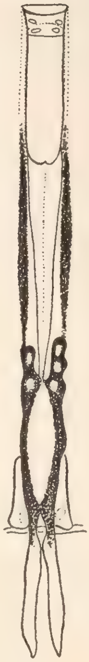

2

EXPLANATION OF FIGURES

con. = cone
b.m. = basement membrane
d.p.c. = distal pigment cell
p.p.c. = proximal pigment cell
rh. = rhabdome
r.p.c. = reflecting pigment cell

FIG. 1. An ommatidium of a typical light-adapted eye showing the positions of the eye pigments. This and the following figures of ommatidia show the situation as seen in thin sections of the eye. In the intact light-adapted eye each cone, cone process and rhabdome is almost completely surrounded by a cylinder of pigment.

FIG. 1*a*. Showing the appearance of an intact eye with the pigment distribution seen in Fig. 1 when viewed, in the dark, by bright reflected light.

FIG. 2. An ommatidium showing the effect on the pigments of injection of eyestalk extract into a light-adapted animal.

FIG. 2*b*. The intact eye has essentially the same appearance as does the normal light-adapted eye.

An ommatidium from a typical eye of a light-adapted animal, injected with eye-stalk extract and left in the light, is represented by Fig. 2. The distal pigment is in a more extreme proximal position and all of the proximal pigment is above the basement membrane. It is as though the effect of the injected material were added to the effect of light, which probably acts by causing the release of the active material or hormone. The intact eye of such an animal has essentially the same appearance as does the normal light-adapted eye (Fig. 2*b*), although it may not be as black. This is due to the distance of the distal pigment from the surface of the eye.

IV

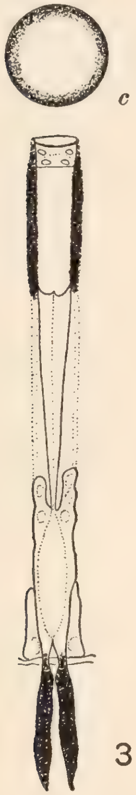
When specimens of *C. bartoni* were placed in the dark, the typical dark-adapted condition in the eye was seen after two hours or less. It is known, however, that there is a diurnal migration of proximal retinal pigment in crayfishes which are kept in continuous darkness (Bennitt, 1932), so in order to assure uniform conditions in all experiments on dark-adapted animals the majority of observations were made in the early evening.

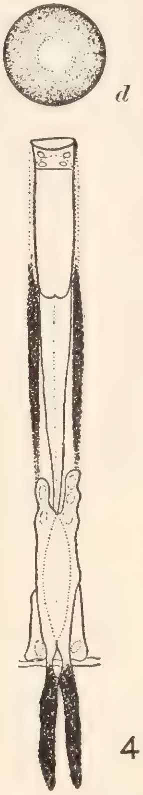
The positions normally occupied by pigments in a dark-adapted eye are shown in Fig. 3. The distal pigment forms a collar surrounding the cone and the proximal pigment is all below the basement membrane. In such a condition the rhabdome of a given ommatidium may receive light from neighboring ommatidia. Such an eye is referred to as a superposition eye and is commonly found in those insects and crustaceans which are active at night.

The intact eye of a dark-adapted crayfish has a brilliant orange-red center when viewed by reflected light, due to the mirror-like property of the exposed reflecting or tapetal layer (Fig. 3*c*). The color is due to the visual red of the rhabdomes.

When dark-adapted *C. bartoni* were injected with eye-stalk extracts, and left in the dark, varying effects on the pigment were seen depending on the amount injected, and the interval between the time of injection and the time of observation. Animals which were dark-adapted for several hours and injected in the early evening with an amount of material equivalent to that obtained from one-fourth to one eye-stalk showed, after three hours, a migration of the distal pigment to or toward the light position. The proximal pigment was not affected (Fig. 4). When such eyes are viewed by reflected light they appear gray rather than black and very little light is reflected from the tapetal layer (Fig. 4*d*).

The injection of 0.1 cc. of the extract (= the extractible material from two eye-stalks) had a distinct effect on the proximal as well as the distal pigment. After three hours both pigments were found to occupy positions more or less typical of light adaptation (Fig. 5). The intact eye when viewed by reflected light had the same appearance as the normal light-adapted eye (cf. Fig. 5*e* with 1*a*).





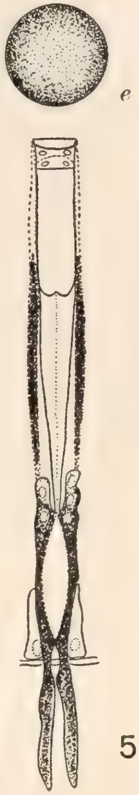


FIG. 3. Ommatidium of a typical dark-adapted eye showing the positions of the pigments.

FIG. 3*c*. The intact eye of a dark-adapted animal has a bright orange-red center when viewed by reflected light.

FIG. 4. The injection of the active material from one eye-stalk into a dark-adapted animal causes the migration of the distal pigment to the light position.

FIG. 4*d*. The intact eye of such an animal may have a small reflecting central area.

FIG. 5. The injection of the active material from two eye-stalks into a dark-adapted animal causes the migration of both distal and proximal pigments to their light positions.

FIG. 5*e*. The intact eye of such an animal has the same appearance as that of a light-adapted animal.

V

It has been demonstrated that it is possible, by means of a simple extraction process, to obtain from two eye-stalks of a crayfish an amount of retinal pigment activator, or hormone, equivalent to that normally released by the animal during the process of light adaptation. From one eye-stalk the amount of hormone is sufficient only to activate the distal pigment cells; thus indicating that they have a lower threshold than do the proximal pigment cells. Such threshold differences between the three sets of pigments in a given species may account for such a situation as was first seen in the eye of *Macrobrachium* (Welsh, 1930), where under continuous illumination the distal pigment cells migrate toward the periphery of the eye at the time of sunset and return to a proximal position at the time of sunrise, while the proximal pigment remains in a constant light position (see also Welsh, 1935, 1936; and Kleinholz, 1937, 1938).

The injection of eye-stalk extracts into dark-adapted crayfishes makes it possible to obtain a "light-adapted" eye, as regards the positions of the screening pigments, while the "dark-adapted" level of the light-sensitive substance of the retina remains unaffected. This enables one to study the effect of pigment position on visual acuity and response to flicker and has been employed by Crozier and Wolf (1939).

SUMMARY

A substance similar to, or identical with, the eye-stalk or chromatophorotropic hormone may be obtained from the eye-stalks of crayfishes. When injected, in proper amount, into light-adapted crayfishes it causes the distal and proximal pigments to migrate to more extreme "light positions" than normal. When injected into dark-adapted crayfishes which are allowed to remain in the dark it causes the migration of one or both sets of screening pigment to their "light positions." The distal pigment has a lower threshold than the proximal pigment, as it is affected by lower concentrations of the active substance. It is suggested that such threshold differences may account, in part, for the unusual pigment responses which have been observed in compound eyes in studies of 24-hour cycles in pigment migration.

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THE FLICKER–RESPONSE CONTOUR FOR THE CRAYFISH

II. Retinal Pigment and the Theory of the Asymmetry of the Curve

W. J. CROZIER AND ERNST WOLF

(From the Biological Laboratories, Harvard University, Cambridge)

I

The flicker-response contour ($F - \log I$) for the crayfish *Cambarus bartoni* resembles that for other arthropods having markedly convex eyes (see Crozier and Wolf, in press). Only its very uppermost part can be fitted by a probability integral. Over its lower part the slope increases too rapidly, so that the whole curve is quite asymmetrical. This departure from the rule observed in the responses of vertebrates (see Crozier and Wolf, 1937*a* and *b*, 1938*a*) has been accounted for (Crozier and Wolf, 1937*c*, 1938*b*) by the shape of the optic surface in the majority of arthropods. With increasing flash-intensities the retinal area effectively involved is increased, which results in a higher F; this is due to the greater chance of exciting ommatidia toward the circumference of the curved eye. Confirmation of this view, consistent with the consequences of changing the light-time fraction in the flash-cycle (Crozier and Wolf, 1937*c*, 1938*b*), is given by the fact that an arthropod with sufficiently flat optic surfaces, the isopod *Asellus* (Crozier and Wolf, 1939), gives a flicker-response contour which is a perfectly symmetrical probability integral. The asymmetry of the curve with *Anax* is appropriately reduced by blocking out all but a central area of the eye (Crozier and Wolf, 1937*c*, 1938*b*), and in a form with still more markedly curved optic surfaces (*Cambarus*) (see Crozier and Wolf, in press) the asymmetry is much more extreme.

In our experiments with *Anax* (Crozier and Wolf, 1937*c*, 1938*b*) the limitation of the increase of effective retinal area with increase of illumination by painting portions of the eyes was recognized to be imperfect. A certain amount of leakage of light near the margins of a cap of enamel, and under its edge, cannot be prevented. A neater method of accomplishing the purpose is to use the migrations of retinal pigment cells. The flicker-response contours we have discussed were determined with animals previously dark-adapted. For such a crustacean as *Cambarus* this means that the proximal retinal pigment is below the level of the receptive retinulæ, the distal pigment cells well out toward the surface of the eye around the crystalline cones. The retinulæ are completely unshielded from laterally spreading light, and the condition is that for the "superposition" type of eye (Exner, 1891). In the eye well light-adapted the forward migration of the proximal pigment shields the retinulæ, while the inward movement of the distal pigment forms around each ommatidium an opaque tube of pigment along the length of the crystalline lens and down to the proximate pigment (Bernhards, 1916; Day, 1911; Parker, 1932). The effective isolation of each recipient unit from light other than that proceeding down the axis of the ommatidium then produces the condition for the "apposition eye" (Exner, 1891).

For our purposes, however, no use could very well be made of the control of retinal pigment migration by light. The process of light adaptation involves not only movements of the retinal pigment cells, but also, it must be presumed, the intrinsic photic adaption of the visual response system itself. At the same time, if some other procedure could be found to cause the retinal melanophores to assume the "light-adapted" condition, it should serve admirably for a test of certain properties of the *Cambarus* flicker-contour. It should also give some direct behavioral evidence as to the functional rôle of the retinal pigment and its movements, as well as providing material for a logical approach to the method of estimating the time-course of visual light-and-dark-adaptation in such animals.

It was pointed out to us by Dr. J. H. Welsh that extracts containing the "eyestalk hormone" from the optic peduncle produce an effect on the melanophores and also on the movement of retinal pigment (Kleinholz, 1934, 1936, 1938; Welsh, 1939) in dark-adapted eyes of *Cambarus*, so that injection of sufficient extract into a dark-adapted animal leads to the migration of retinal pigment into positions characteristic of the normal light-adapted state. This we have verified in *C. bartoni.*

II

The observational procedure was identical with that employed in measuring the flicker-response contour for dark-adapted *Cambarus* (Crozier and Wolf, in press): temperature 21.5°, 50 per cent light-time in the flash cycle. To keep the handling of the animals uniform with respect to time after injection and the like, a lot of 5 rather than of 10 was used. The eyestalks from 10 *Cambarus bartoni* were extracted in Ringer solution. Into each crayfish prepared for observation there was injected into the abdomen 0.08 ml. of extract, the equivalent of 2 eyestalks. After 75 to 90 minutes in the dark the crayfish are bluish in body color and by means of a beam of light directed into the eye the retinal pigment is seen to be in the position characteristic of light adaptation. Sectioned eyestalks fixed in hot water at this stage show the condition clearly under the ultrapak microscope. In the normal dark-adapted eye the proximal pigment is retracted below the basement membrane, while the distal pigment is out between the crystal cones. There is no detectable pigment between the ommatidial units. After about 90 minutes in darkness subsequent to injection of eye-stalk extract, the proximal pigment surrounds the retinulæ, while the distal pigment now envelopes each ommatidial unit down to its base. The condition is one of quite complete shielding of each ommatidium by a dense layer of black pigment, more extreme than is the case in ordinary light adaptation.

TABLE I

Data for the flicker-response contour of the crayfish *Cambarus bartoni*, with eye-pigment in the "light adapted" state as result of injection of eye-stalk hormone. $N = 5$ individuals, $n = 3$ observations on each; the same individuals used throughout; $t° = 21.5°$ C.; $t_L = t_D$. See Fig. 1. I in millilamberts, F in flashes per second. P.E.$_1$ = P.E. of the dispersions.

F	F_m	$P.E._{1F_1}$	log I_m	log $P.E._{1I_1}$
2			$\bar{3}.3404$	$\bar{5}.9958$
5			$\bar{2}.0777$	$\bar{4}.3334$
8			$\bar{2}.2584$	$\bar{4}.4806$
12			$\bar{2}.5249$	$\bar{4}.8155$
16			$\bar{2}.6674$	$\bar{4}.9737$
20			$\bar{2}.7771$	$\bar{3}.0398$
25			$\bar{2}.9254$	$\bar{4}.8291$
30			$\bar{1}.0730$	$\bar{3}.2355$
35			$\bar{1}.2865$	$\bar{3}.6169$
40			$\bar{1}.5937$	$\bar{3}.8785$
	41.79	0.289	0.00	
42			0.0233	$\bar{2}.3664$
43			1.0077	$\bar{1}.2823$
	43.10	0.357	0.50	
	43.23	0.361	1.00	
	43.66	0.282	1.25	
44			1.5231	0.1464
	44.05	0.130	1.50	

III

The determinations of mean critical flash-intensity and mean critical flash-frequency for response (Crozier and Wolf, in press) to visual flicker are given in Table I. Comparison with the results for normally dark-adapted *Cambarus bartoni* (Crozier and Wolf, in press) shows that there is a pronounced (reversible) effect of the injection of eyestalk hormone upon the properties of the flicker-response contour. This cannot reasonably be traced to an effect of the eye-stalk extract upon the intrinsic processes of photic excitability, for several reasons. In the first place injection of *ca.* 0.06 ml. of the eyestalk extract into *Anax* (dragon fly) nymphs produces no detectable effect either on pigment migration or on the flicker-response curve, as the following observations showed (tests on 5 individuals):

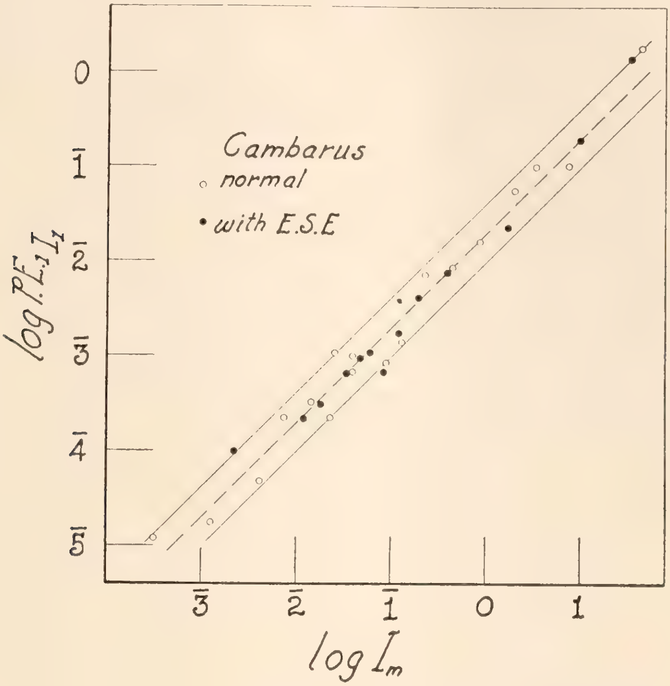

FIG. 1. The variation of I_1 for normal *Cambarus bartoni* and after injection with eye-stalk extract (*E.S.E.*); Table I; see text.

F	Normal log I_m	Normal + eye-stalk extract log I_m
20	$\bar{2}.473$	$\bar{2}.478$
30	$\bar{2}.749$	$\bar{2}.745$
	$\bar{2}.741$	$\bar{2}.750$

Any effect of this sort would thus have to be specific. In the second place, the results of adapting *Cambarus* are rapidly apparent even when the retinal pigment is already fully advanced into the "light" position, as subsequently shown (§ IV). Finally the various modifications of the flicker-response contour are those to be expected as the result of the optical shielding of the ommatidia, so that no specific effect on excitability need be invoked.

For any given level of flash-intensity the variation of I_1 among the individuals used is statistically of the same magnitude as for the normal group previously examined (Crozier and Wolf, in press). The 5 individuals giving the data of Table I were in the lot of 10 providing the normal curve for this species (Crozier and Wolf, in press). The scatter of the variation indices (P.E.$_{I_1}$) is even a little less than might have been expected in view of the smaller number of readings in the eyestalk injection series (Fig. 1).

The effects to be expected if the "dark" position of the retinal pigment shields ommatidia from all but light parallel to the retinular axis, and if this is to prevent the recruitment of optic impulses from a larger retinal surface as flash intensity is increased, are the following: (1) the total achievable sensory effect ($= F_{max.}$) must be reduced; (2) at given I, F must be less; (3) the asymmetry of the $F - \log I$ curve must be markedly reduced; and (4) it would not be surprising to find the slope of the "fundamental" curve increased (i.e., $\sigma'_{\log I}$, for the ideal frequency distribution of log I thresholds, *reduced*), owing to the mechanical exclusion of a large proportion of the otherwise marginally excitable units.

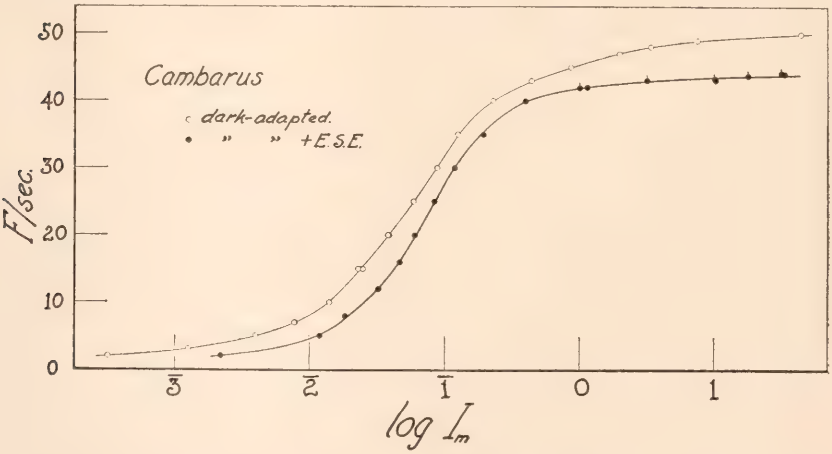

FIG. 2. F—log I curves for dark-adapted *Cambarus* and under the same conditions for individuals injected with eye-stalk extract (*E.S.E.*); Table I.

Figure 2 shows that the $F - \log I$ curve with *Cambarus* dark-adapted but under the influence of eye-stalk extract is moved toward higher intensities and exhibits a lower maximum. These are the

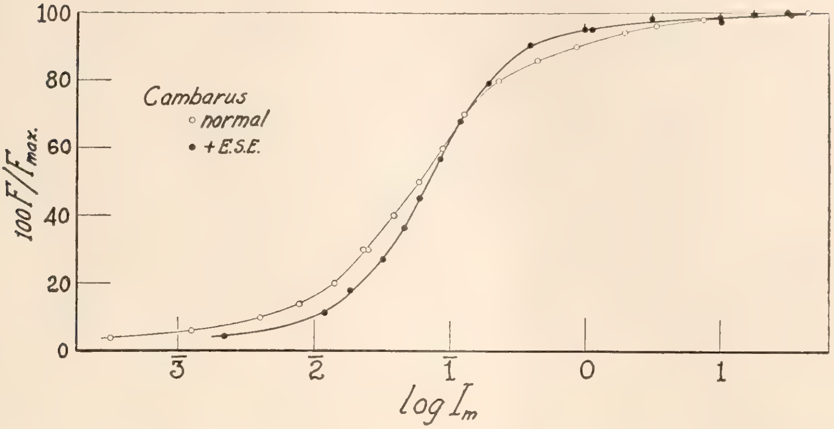

FIG. 3. The curves of Fig. 2 brought to the same $F_{\max.}$ (= 100 per cent), to show change of shape.

results of a decrease in the total number of excitable elements (Crozier and Wolf, 1937*c*, 1938*b*), as expected.

The asymmetry of the curve is also decreased (Fig. 3). The

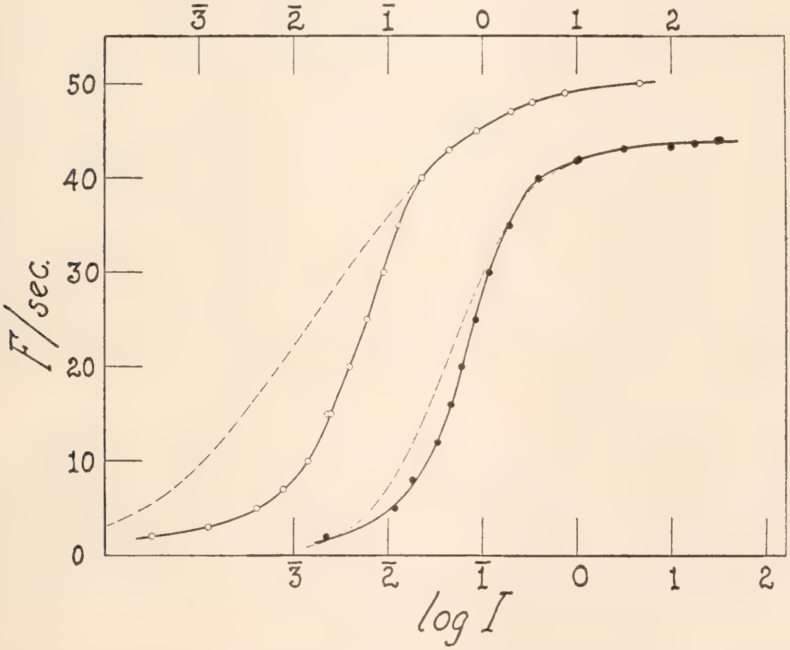

FIG. 4. The curves of Fig. 2 with probability integrals adjusted to the upper portions (*cf.* Crozier and Wolf, 1937*c*, 1938*b*, 1939, and paper in press), to show that the flicker-response contour after injection of eye-stalk extract departs less than the normal; see text.

sheathing of the ommatidia by pigment materially reduces the chance of photic action on additional elements as intensity increases, hence the slope of the $F - \log I$ curve cannot increase so rapidly.

It is to be presumed that in the absence of comparatively free passage of light through the eye (as in the dark-adapted state), the actual intensity at each receptor locus will be decreased. This cannot be a major factor in the changes shown in Fig. 2, else increase of intensity would find the $F - \log I$ curve continuously rising at its upper end.

The diffusion of light within the substance of the eye cannot be ignored, however. Figure 4 shows that the asymmetry of the flicker-response contour has been decreased (*cf.* Fig. 3), but not abolished. In view of the proximal movement of the distal retinal pigment under the influence of eye-stalk extract (Kleinholz, 1934, 1936, 1938; Welsh, 1939), this is not surprising. It probably explains the slight but detectable rise of the curve at the highest intensities used (Table I; Fig. 2), particularly when F_m is determined at constant flash-intensity; this cannot be accounted for by light adaptation (§ IV).

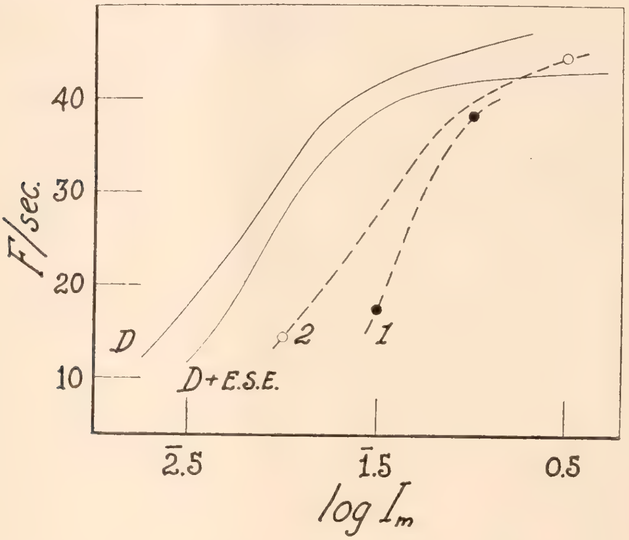

FIG. 5. Portions of the curves in Fig. 2 for dark-adapted *Cambarus* with (D + $E.S.E.$) and without (D) injection of eyestalk extract, and the first (1) and second (2) sets of readings (Table II) during the progress of dark adaptation after light adaptation.

With allowance for this effect, a reasonable adjustment of an ideal probability integral can be made to the upper part of the curve (Fig. 4).

Comparison with the normal, in the same figure, shows that $\sigma'_{\log I}$ is, as expected, much reduced.

IV

Light adaptation of *Cambarus* reduces the $F - \log I$ curve (Table II, Fig. 5); with even brief residence in darkness the curve rises toward the position typical for dark adaptation (Fig. 5). Obviously, for a quantitative investigation of the kinetics of photic excitation, the effect of the migration of retinal pigment as governed by light and darkness must be ruled out. The present data supply the first evidence of a functional rôle of the position of the retinal pigment in matters of visual response. The result of light adaptation, as with certain other forms, is to reduce F at fixed I, but to follow by this means the recovery of excitability during subsequent darkness is made difficult by the fact that the retinal pigment also changes position. Either the pigment must be held in a fixed position throughout, by suitable repeated injection of eye-stalk extract, or else a procedure found for extrapolation to a constant condition of the pigment.[1] The latter could perhaps be achieved by determining the relation between the position of the $F - \log I$ curve and various known positions of the pigment; in any event the whole course of the function must be known.

TABLE II

Critical flash-frequencies at two flash-intensities for *Cambarus:* (1) very shortly after light adaptation to bright daylight; (2) after *ca.* 10 minutes in darkness; 3 observations on each of the same 4 individuals at all points; 21°.5, $t_L = t_D$. See text, and Fig. 4.

	log I	F_m	P.E.$_{1F_1}$
(1)	$\bar{1}.50$	**17.5**	0.491
	0.00	**38.3**	0.371
(2)	$\bar{1}.00$	**14.5**	1.52
	0.50	**44.5**	4.81

SUMMARY

Injection of *Cambarus bartoni* with extract of eyestalks of this species forces migration of retinal pigments of individuals kept in darkness into positions characteristic of the light-adapted eye. In this condition the receptor elements of each ommatidium are effectively shielded from light passing through their neighbors. The flicker-response contour then differs in four particulars from that found when the retinal pigment is in the "dark" position, for which effective screen-

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[1] For a somewhat analogous case of changing sensitivity during the interval of observation, a technic of this kind was used with *Agriolimax* (Crozier, W. J., and Wolf, E., 1928–29, *Jour. Gen. Physiol.*, **12**: 83).

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ing of the ommatidia is not present: $F_{max.}$ is lowered; the whole curve is moved to higher intensities; the spread of the log I thresholds for the cumulative population of sensory effects is lessened; and the asymmetry of the $F - \log I$ curve is markedly reduced. It is pointed out that these results are to be expected if the asymmetry of the curve in normal dark-adaptation is due to the relation between flash-intensity and the curvature of the optic surface and divergence of the ommatidial axes.

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THE SIGNIFICANCE OF GERMARIA IN DIFFERENTIATION OF OVARIOLES IN FEMALE APHIDS

CHESTER A. LAWSON

(*From the Department of Biology, Wittenberg College, Springfield, Ohio*)

INTRODUCTION

Recognition of the fact that winged parthenogenetic female aphids produce both parthenogenetic and gamic female offspring differing in part in the structure of the ovarioles, invites an understanding of the mechanism that controls the development of the ovarioles. Such an undertaking may aid in determining how genes, presumably identical, can produce two types of individuals.

In studies on the development of aphids (Lawson, 1939), it was noted that the germaria are the first embryonic structures to mark a distinction between gamic and parthenogenetic females. Other differentiating characters do not appear until after birth. As determination of all differentiating characters occurs before birth (Shull, 1930*a*), it is possible that the germaria are instrumental in determining the adult nature of the individual at least in so far as the ovarioles are concerned.

In female aphids the essential reproductive organs consist of a pair of ovaries in which the eggs are developed, and an oviduct leading from each ovary to an external opening. Each ovary is made up of a number of loosely parallel ovarian tubes (ovarioles) which open into the oviduct. Three different regions are recognized in an ovariole (Fig. 1),—the terminal filament, the germarium, and the vitellarium. The terminal filament is a thread-like structure at the end of the ovariole farthest from the oviduct, which attaches the ovariole to the body wall. Next behind the terminal filament is the germarium which contains the germ cells from which the eggs develop, and nurse cells whose function is to furnish nutriment to the developing eggs. The vitellarium is a tubular structure which extends from the germarium to the oviduct and contains developing eggs in a gamic female and both eggs and embryos in a parthenogenetic female. A nutritive thread or yolk stream extends from the nurse cells to the youngest growing oöcyte in the vitellarium.

DIFFERENCES BETWEEN GAMIC AND PARTHENOGENETIC FEMALES

Gamic females are described by Shull (1930*b*) as follows: "Gamic females of this species of aphid have a wax yellow body color, dark

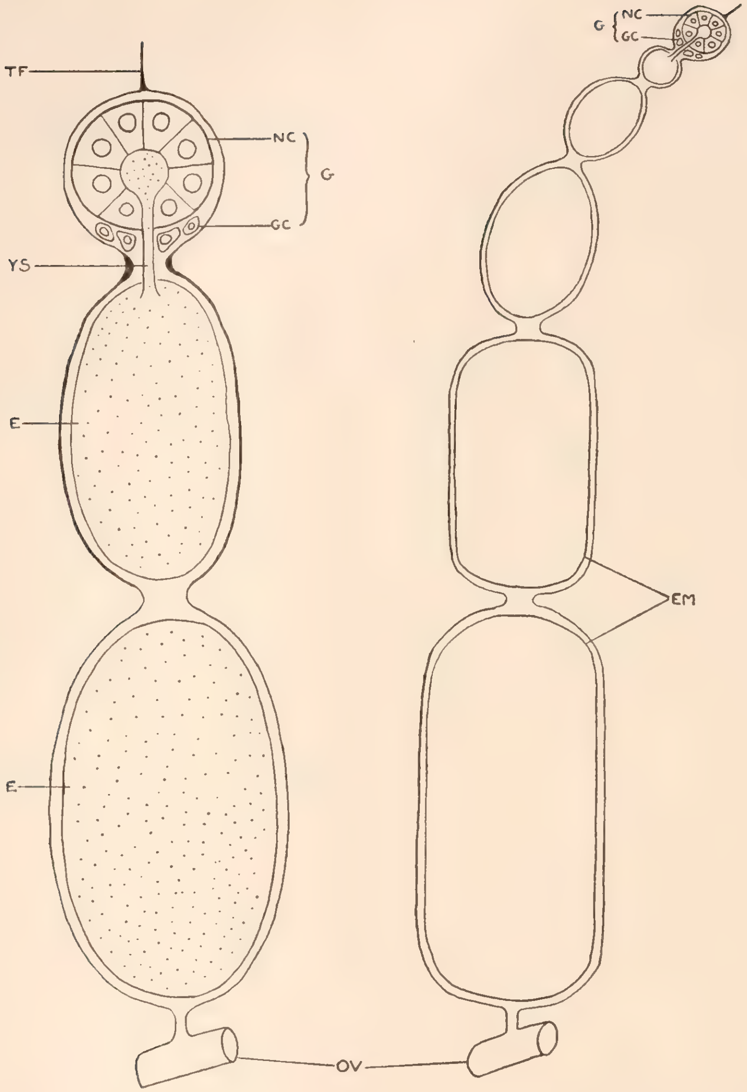

FIG. 1. Diagrams of aphid ovarioles, gamic at left, parthenogenetic at right. *E*, egg; *Em*, embryo; *G*, germarium; *GC*, germ cell; *NC*, nurse cell; *OV*, oviduct; *TF*, terminal filament; *YS*, yolk stream.

brown antennae, and greatly swollen hind tibiae of dark brown color, covered with hundreds of sensoria. Their reproductive systems consist of a vagina on which are borne a pair of colleterial glands and a seminal receptacle, a pair of short oviducts formed as branches of the vagina, and a variable number (usually ten) of ovarioles branching from the oviducts. Each ovariole, in a mature female, contains usually one mature or nearly mature egg, distinctly opaque and of very regular ovoid form; beyond this often an oöcyte, in early growth stage, hence long and slender and not very opaque; and lastly a large, spherical germarium forming a conspicuous knob at the end of the ovariole. In old gamic females, especially those that have not been laying eggs, the second reproductive cell from the base of the ovariole may be large and opaque and regular in form, and is then presumably mature like the one posterior to it. Almost never, however, in typical gamic females (that is, those produced at low temperature by winged females whose other daughters are practically all gamic), are there more than two oöcytes in any stage in one ovariole. An ovariole of a gamic female may therefore be regarded as regularly consisting of a tube containing one or two eggs or oöcytes, and a large round germarium."

In stained sections of adult gamic females the ovarioles (Fig. 1) are prominent in the abdominal cavity. The germaria usually lie anterior to the large yolk-laden eggs. Each germarium is surrounded by the closed end of an ovariole tube composed of a single layer of thin, squamous epithelial cells. Posterior to the germarium the tube constricts, forming a short neck in which the lumen is quite narrow. The narrowing of the lumen is due in part to contraction of the tube, but also to an increase in the height of the cells which change from a squamous to a columnar type in the neck region. A constriction of the tube likewise occurs between the eggs contained in the vitellarium, but this is due to contraction only as there is no change in cell shape. Around the young growing oöcytes the ovariole wall is constructed of a single layer of cuboidal cells. In the posterior part of the ovariole the cells are rectangular with the greatest width parallel to the surface of the egg.

The germarium is composed of two types of cells, a round ball of large nurse cells and a small group of germ cells. Each nurse cell is roughly pyramidal in shape (triangular in section) with the base at the periphery of the germarium and the apex in the center. The nurse cells fit closely together and form a ball, in the center of which is a substance secreted by the nurse cells. This substance flows from the central area through the neck of the ovariole into the growing oöcyte within the vitellarium. The exact nature of this substance is unknown, however, as it flows directly into the growing oöcyte; it might be yolk. No better term is available, hence the term "yolk" is used to facilitate discussion, and the term "yolk stream" is used to indicate the distinct cord, or string of substance which passes from the germarium into the growing oöcyte.

Germ cells, smaller and fewer in number than the nurse cells, lie between the ball of nurse cells and the neck of the ovariole. Posterior to the neck the vitellarium contains ovoid oöcytes in various stages of growth. Because only young adults were used for this study, it is likely that no mature eggs were examined. Each oöcyte consists of a central mass of yolk surrounded by a thin peripheral layer of cytoplasm. Outside of this cytoplasm is a single egg membrane. In the smaller younger oöcytes the nucleus occupies the center of the cell, but in older ones it lies at the periphery halfway between the two ends of the egg. The nucleus is large and clear and contains but a few small bodies which stain heavily.

Parthenogenetic females are described by Shull (1930*b*) as follows: "The parthenogenetic females have bright green body color, antennae quite pale except in the distal segment, and very slender, pale hind tibiae bearing no sensoria. The reproductive system consists of a vagina, without collegerial glands or seminal receptacle, two short oviducts branching from the vagina, and a variable number (apparently up to ten) of ovarioles branching from the oviducts. Each ovariole is a very delicate tube containing, in healthy individuals, usually six to nine embryos or eggs or oöcytes, and bearing at the end a very small germarium which is usually not much larger, and is often smaller, than the oöcyte or egg next behind it. All of these reproductive elements except one (the one next to the germarium) are as a rule embryos in some stage of development. They are all translucent or transparent, unless dead, and if dead they have a clouded appearance not at all like the opaque gamic eggs. Only the smaller embryos are ellipsoidal; the medium and larger ones always possess angles which correspond to the form of the young aphids. The six to nine embryos or eggs in one ovariole, in a typical healthy female, are of regularly decreasing size from oviduct to germarium, so that they resemble a tapering string of beads."

In stained sections of parthenogenetic female aphids the entire abdominal cavity is crowded with embryos of varying sizes and stages of development. In general, the larger more developed embryos lie in the posterior region while the smaller less developed embryos lie anterior to them. The germaria (Fig. 1), small and difficult to locate, usually lie in the anterior abdominal region squeezed among young embryos or between the embryos and the lateral body wall. The wall of the ovariole tube around the germarium is a single squamous epithelium which constricts, forming a neck just posterior to the germarium. The cells become somewhat cuboidal in this region. Extending posteriad from the neck the ovariole wall encloses embryos, and is composed of a thin single layer of squamous cells. The tube is always constricted between the embryos within any one ovariole. The parthenogenetic germarium consists of two types of cells, a round ball of nurse cells and a small group of germ cells. Each nurse cell is roughly pyramidal in shape with the base at the periphery of the germarium and the apex in the center. The nurse cells fit closely together and form a ball. This ball of nurse cells contains yolk, and a yolk stream extends from the nurse cells through the ovariole neck into the youngest growing oöcyte. The germ cells lie in the germarium between the ball of nurse cells and the neck of the ovariole. Young oöcytes are found immediately posterior to the germarium. Progressing caudad, the next in line is usually an egg in cleavage followed in turn by a young developing embryo. Thereafter each succeeding embryo is larger and more fully developed.

Major Differences between Adult Gamic and Parthenogenetic Ovarioles

The primary difference between gamic female and parthenogenetic female ovarioles is in the development of the germ cells. In gamic female ovarioles development of germ cells consists of growth through accumulation of yolk and possibly meiosis, though no divisions have been observed in this species of aphid. In the parthenogenetic female ovarioles the germ cells are stimulated to develop parthenogenetically.

The size difference in the germaria presumably is secondary to and correlated with the germ cell difference. Gamic female ovarioles contain eggs that undergo embryonic development outside of the mother's body, are dependent on a large yolk supply and consequently accumulate this yolk supply during growth in the ovariole. The large size of the gamic female germarium (about three times larger than a parthenogenetic female germarium in fixed material) is evidently correlated with the necessity of producing much yolk. Eggs produced by parthenogenetic females develop within the body of the mother, and it is probable that nourishment for this growth and development is supplied directly by the mother by body fluid. Thus a large quantity of yolk is unnecessary for parthenogenetic eggs, and the small size of parthenogenetic female germaria may be correlated with this decreased secretory activity.

Ovarioles of Gamic Female and Parthenogenetic Female Embryos

The ovarioles of gamic female and parthenogenetic female embryos were studied and compared in those embryos of both types which showed the greatest degree of development. These embryos were well developed, occupied the posterior abdominal region of the mother and, presumably, would have been born very shortly had the mother been allowed to live.

Each ovariole in gamic embryos of this late stage of development consists of a terminal filament, a germarium and a vitellarium (Fig. 2). Each germarium contains nurse cells and germ cells. The nurse cells are roughly pyramidal in shape and form a ball at the end of the ovariole. This is similar to the adult condition although the cells seem to be more loosely packed and the entire germarium is more elongated than in the adult. The center of the ball of nurse cells contains yolk, but there is no yolk stream. Germ cells occupy the region of the germarium posterior to the nurse cells. The ovariole tube surrounds the germarium as a simple squamous epithelium and continues posteriad as a small tubular vitellarium with a narrow lumen. The vitellarium contains no eggs or oöcytes.

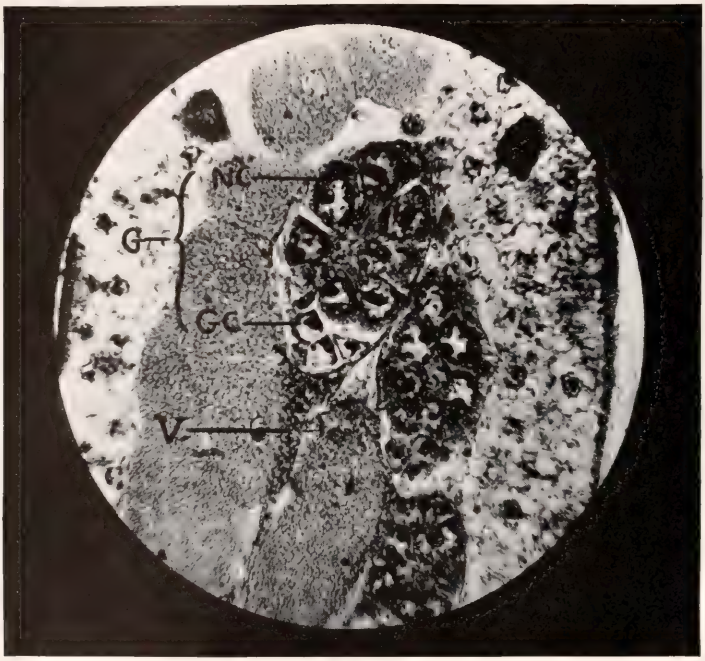

FIG. 2. Photomicrograph of an embryonic ovariole of a gamic female aphid. *G*, germarium; *GC*, germ cell; *NC*, nurse cell; *V*, vitellarium.

In parthenogenetic female embryos of the same degree of development the ovariole consists of a terminal filament, a germarium, a vitellarium, and parthenogenetically developing germ cells (Fig. 3). Each germarium consists of nurse cells and germ cells. The nurse cells form a ball of cells at the tip of the ovariole tube within which is found a small amount of yolk. A yolk stream extends from the center of the ball of nurse cells through the neck of the ovariole into the youngest growing oöcyte. The germ cells lie just behind the nurse cells. Each germarium is surrounded by the closed end of the ovariole tube which consists of a thin squamous epithelium. The ovariole tube continues posteriad of the germarium as a thin-walled vitellarium which invariably contains a growing oöcyte just behind the germarium and an egg undergoing cleavage behind the oöcyte.

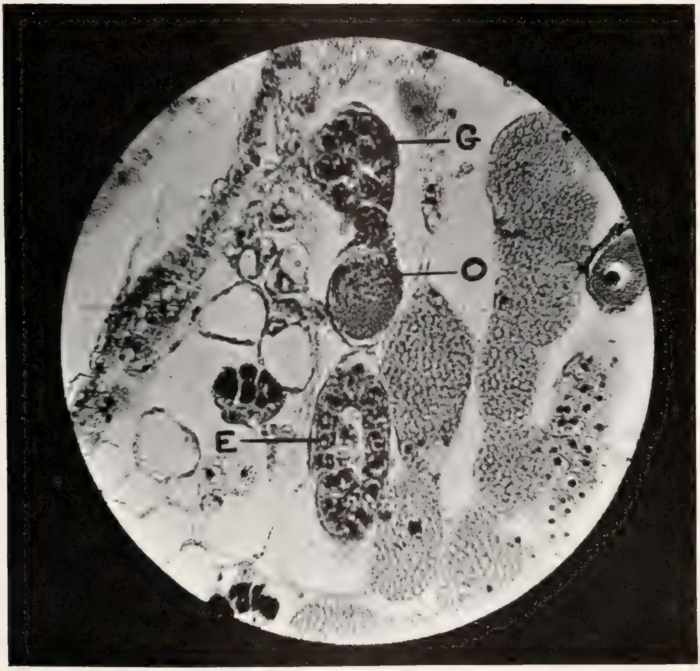

FIG. 3. Photomicrograph of an embryonic ovariole of a parthenogenetic female aphid. *E*, egg undergoing cleavage; *G*, germarium; *O*, oöcyte.

To insure that embryos identified as gamic females were actually gamic, parthenogenetic winged females were fixed when they were producing gamic females only. The parthenogenetic female embryos were studied only in wingless parthenogenetic females which seldom produce gamic females. The offspring of both types of parents produced before fixation were reared and examined.

Do Germaria Control Differentiation of Ovarioles?

The possibility that germaria play a significant rôle in the differentiation of ovarioles is indicated by three observations. (1) Adult ovariole differences are due primarily to the type of egg development that occurs within the ovariole and to the size of the germarium. (2) Germaria contain and produce the germ cells that develop within the ovarioles. (3) Germaria are the first reproductive structures to be differentiated in the embryo (Lawson, 1939).

Determination of an embryo into either a gamic or parthenogenetic female must occur sometime during parthenogenetic development between the growth of the oöcyte and the differentiation of the germaria. This determination may affect the embryo in two ways. (1) It may include the entire embryo in its effect so that the differentiation of the germaria would simply represent the first differential reaction of a general condition throughout the embryo. This would exclude the germaria from any significance in future development. (2) It may occur at the time that germaria are developing and affect them only. According to this assumption the embryo would be potentially capable of developing into either a gamic or a parthenogenetic female prior to determination of the germaria. After this event the aphid (more specifically the ovarioles) would become gamic or parthenogenetic depending on the nature of the germaria.

The study of normal gamic and parthenogenetic female aphids offers no choice between the two ways in which determination may occur. However, aphids intermediate between gamic and parthenogenetic are occasionally produced and an analysis of these intermediates gives us a choice.

These intermediates, described by Shull (1930*b*), show the intermediacy in several structures of which the ovarioles only are to be considered here. The intermediacy is expressed in the ovarioles in a very irregular fashion so that no two aphids with intermediate ovarioles are necessarily identical. An intermediate aphid may be a mosaic with respect to the ovarioles, in that one or more of the ovarioles are strictly gamic while the others are strictly parthenogenetic. Any one ovariole may be intermediate in that the germaria are smaller than normal gamic germaria, but larger than normal parthenogenetic germaria. The contents of the vitellarium may be intermediate in three different ways. (1) Eggs may vary from gamic in being less opaque than strictly gamic eggs. (2) Gamic eggs may occur in greater numbers than is normal for a gamic ovariole. (3) Embryos characteristic of parthenogenetic ovarioles may be abnormal. According to Shull's description, it is possible to have any combination of the above conditions in one aphid.

The first type of intermediate mentioned here in which one individual contains both gamic and parthenogenetic female ovarioles could not be produced unless the ovarioles are able to develop within the aphid independently of one another. As the germaria appear in the embryo prior to other ovariole structures, it follows that the germaria also must develop and be determined independently of one another.

The second type of intermediate in which the germaria are intermediate in size between gamic female and parthenogenetic female germaria indicates that the mechanism of determination is such that intermediate germaria are determined and differentiated within the embryo as well as germaria that are strictly gamic or parthenogenetic.

The mechanism of determination suggested by Shull (1930*a*), in which a high level of some substance within the embryo produces one type while a low level of the same substance produces the opposite type, could very easily account for intermediate germaria. These intermediate germaria could result from a condition in which determination of the germaria occurred when the level of concentration of the determining substance was intermediate between the high and low extremes.

The remaining types of intermediate aphids show the intermediacy in the ovariole contents. All of these can be explained by assuming that the germarium attached to the end of each intermediate ovariole is intermediate. As gamic female germaria produce much yolk while parthenogenetic female germaria produce little yolk, it would be expected that intermediate germaria would produce an amount of yolk intermediate between the gamic and parthenogenetic extremes.

One of the types of intermediate ovarioles described by Shull had eggs that were gamic but less opaque than strictly gamic eggs. Such eggs could be produced by a germarium that was gamic with respect to the type of eggs produced but intermediate with respect to yolk production. The decreased amount of yolk in the eggs might make them less opaque than normal gamic eggs.

Another type of intermediate ovariole had gamic eggs in greater numbers than the typical one or two of strictly gamic ovarioles. One characteristic of gamic germaria is that they produce no more than two eggs while parthenogenetic germaria produce many more than two. The above intermediate ovariole could have resulted from a germarium that was intermediate with respect to the number of eggs produced, while at the same time it was gamic with respect to the type of eggs produced.

The last type of intermediate described by Shull contained parthenogenetic ovarioles in which the embryos were abnormal. The details of the abnormality were not described but it is possible that the abnormality could have been due to an intermediate germarium in which parthenogenetic oöcytes or eggs were produced plus a quantity of yolk greater than is normal for a parthenogenetic germarium. This abnormal amount of yolk very likely would interfere with normal embryonic development and produce abnormal embryos.

The theory is proposed that germaria are determined independently of one another and also of the rest of the aphid embryo, that a single germarium may be caused to develop into a gamic female type, a parthenogenetic female type or a type intermediate between the gamic and parthenogenetic types. It is proposed, further, that the germarium once determined, controls the differentiation of the ovariole to which it is attached and thus controls, in part, the development of the adult aphid type.

SUMMARY

Winged parthenogenetic female aphids produce both parthenogenetic female and gamic female aphids.

The ovarioles of adult gamic female and parthenogenetic female aphids differ primarily in the nature of the eggs developing within them and secondarily in the size and secretory activity of the germaria. Gamic female germaria are large and secrete much yolk; parthenogenetic female germaria are small and secrete little yolk.

The ovariole differences apparent in the adult aphids are also evident in the embryos. In parthenogenetic female embryos of a late stage of development the embryonic ovarioles already contain oöcytes and eggs undergoing parthenogenetic development while in the gamic female embryos of the same stage of development the germ cells have not yet entered the vitellarium. The germaria of parthenogenetic female embryos are smaller than the germaria of the gamic embryos of the same stage of development.

In both gamic and parthenogenetic female embryos the germaria are the first reproductive structures to develop.

The theory is proposed that determination of the ovariole type (either gamic female or parthenogenetic female) affects the germaria only. Each germarium, thereafter, controls the development of the ovariole to which it is attached.

Aphids intermediate between gamic female and parthenogenetic female aphids with respect to the ovarioles are described and analyzed to support the above theory.

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ACTIVITY-PREVENTING AND EGG-SEA-WATER NEUTRALIZING SUBSTANCES FROM SPERMATOZOA OF ECHINOMETRA SUBANGULARIS

WALTER E. SOUTHWICK

(*From the Bermuda Biological Station for Research* [1])

Introduction

A spermatozoön, like all highly specialized biological units, has many utterly unique, but very significant characteristics. Of these, probably the most fundamental is the finiteness of its period of life. As has been clearly demonstrated by Lillie (1919), Gray (1928) and others, mature spermatozoa, except possibly those of certain arthropods, are unlike all other biological units in that they do not at any time ingest or otherwise assimilate substances from which energy may be derived. Once, therefore, that a spermatozoön has been liberated from the testis, its every power and activity must be accomplished on the basis of substances that were located in its structure at the time of its liberation. Studies to determine what different substances might be located in a spermatozoön, the location, nature, and approximate amounts of such substances, the changes in location and state which they might undergo, and the factors which might influence, affect, or effect such processes are, therefore, especially important for an understanding of spermatozoan physiology. A series of studies which were made at the Bermuda Biological Station for Research have provided some observations concerning such substances.

Materials and Procedures

The studies were made with the common reef urchin, *Echinometra subangularis*, individuals of which were obtained from the reefs on the eastern end of St. David's Island. The trip to the reefs was made by bicycle, and the urchins were brought to the laboratory in a pail with a small volume of water and covered with seaweed. The water

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[1] Laboratory space for this investigation was provided by the Bermuda Biological Station for Research, Inc. during July, 1938.

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was continually shaken, and so kept reasonably well aerated during the trip, a period of approximately twenty minutes.

Upon arrival at the laboratory, the urchins were immediately transferred to vessels in which a large volume of air was continually bubbled through a comparatively small volume of circulating sea-water. In such vessels, the urchins remained in good condition for approximately three days, and so trips were made regularly every two or three days in order to maintain a constant supply of freshly collected urchins.

The animals were opened by means of the usual circumferential cut, and the body fluids and intestines carefully removed. Every precaution was taken to avoid contamination, either of eggs with sperm, or of the sperm with egg secretions, and the gonads were removed from the test with glass needles. The ovaries were divided into small pieces in a small volume of sea-water with glass needles, and the mass strained through several thicknesses of unbleached cheesecloth. The supernatant fluid from the eggs was removed ten minutes later and fresh sea-water added. After two or three washings in this way, the supernatant fluid was allowed to stand for thirty minutes, at which time it would be capable of producing clear-cut and definite agglutinations with fresh sperm. The egg-sea-water was kept separate from the eggs during all subsequent tests. The testes were removed to a clean, dry Syracuse watch crystal, and, as fresh sperm was needed, the tubules were broken with a glass needle and the exuding dry sperm collected with a moderately fine pipette.[2] The watch crystal with the testes and the containers with the eggs or egg-secretions were kept covered at all times except when the materials were being withdrawn.

MOVEMENTS OF THE SPERMATOZOA

When dry sperm is examined immediately under the microscope, the whole mass may be seen to be in a state of most intense vibratory activity. In such spermatozoa, however, due probably to the compactness of the mass, the active vibrations of the tails serve but to cause a rapid milling about of waving heads. Progressive movements are entirely absent, and the same group of spermatozoa sway back and forth, in constant, rapid vibration, but always in the same position, relative to each other and to the field of the microscope.

In such dry sperm, this motion lasts for from 50 to 120 seconds, and then all movement ceases. The cessation in activity, once it has started, spreads rapidly, so that the entire transition from universal activity to complete quiescence is accomplished within about 15 seconds. Such spermatozoa do not show motion when they are redistributed mechanically by means of pressures on the cover glass.

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[2] Insemination tests made with such spermatozoa in a dilution proportion of one drop of dry sperm to 20 cc. sea-water, one drop of this suspension to one drop of eggs in 7 cc. sea-water gave, consistently, a fertilization percentage of from 92 to 100 per cent.

When, however, a drop of sea-water is brought in contact with a drop of dry sperm, the edge of the drop of dry sperm "frays" slightly. After about one minute, the spermatozoa in this frayed edge begin to move slowly. Gradually, they come to move more rapidly, more spermatozoa become active, and they move slowly out into the drop of sea-water. In about two minutes, the whole mass, or as much of it as is reached by the diffusing sea-water, becomes intensely active. In the denser portions of this mass, as in the original drop of dry sperm, the motion is essentially a rapid milling about of waving heads with progressive movements entirely absent. In the less dense portions, however, progressive movements do occur.

When the dilution of one drop of dry sperm to one drop of sea-water is made upon a glass slide where such changes in the supporting medium as might be produced by evaporation are minimized by means of placing pure petroleum jelly around the edges of the cover glass, the activity continues, with gradually diminishing intensity, for about three or four hours, when all motion ceases. If another drop of sea-water then be added to the suspension under the cover glass, the spermatozoa again become active, and this activity continues with gradually decreasing intensity for about one and one-half hours, when all motion again ceases. Such spermatozoa can be reactivated again by the addition of another drop or two of sea-water, but the length of time during which the spermatozoa remain active with each successive reactivation progressively decreases until finally, after about eight such reactivations, no further activation is obtained. This phenomenon is shown more clearly in Table I which gives the actual observations of a typical experiment of this nature.

If the reactivation obtained from the addition of fresh sea-water to a suspension of inactivated spermatozoa were due to dilution effects, then one might expect a substance to occur in the suspensory fluid of an inactivated suspension of spermatozoa which would have the property of preventing dry sperm from becoming active. In order to test this possibility, a suspension of dry sperm was centrifuged for 30 minutes at 4,000 revolutions per minute. When this centrifuging had been completed, a clear, slightly opalescent, bluish fluid comprising about 11 per cent of the total volume of the original dry sperm had separated from the mass of spermatozoa. Utmost care had been used to have the centrifuge tubes clean and *perfectly dry* before the spermatozoa were added, and great care was now used to have pipettes, glass slides, and cover glasses all perfectly dry. A drop of the clear supernatant fluid was removed from the centrifuge tube and carefully brought in contact with a drop of dry sperm. The whole process of fusion of the two drops was carefully watched with the microscope, but there was no slightest sign of any activation whatsoever at any time. Even when the spermatozoa were thoroughly distributed throughout the supernatant sperm fluid by means of stirring with a

TABLE I

Changes in the activity of a suspension of spermatozoa with time, and the effects of repeated additions of fresh sea-water upon the duration and changes in the activity of the spermatozoa in such suspensions.

Time	Observations on the Activity	Treatment of the Suspension	Number of the Sea-water Additions	Time Interval between Sea-water Additions
10:45		1 dr. dry sperm	0	
10:45	Intensely active			
10:47	All sperm utterly motionless	1 dr. sea-water	1	
10:48	Intensely active			
11:02	Active			
11:13	Some motion			
11:29	Some motion			
11:44	A little motion			
12:05	A little motion			
12:26	A little motion			
1:21	Still a little motion			
1:48	Just a little motion			
2:12	No motion	1 dr. sea-water	2	3 hours 27 min.
2:13	Intensely active			
2:39	Active			
2:49	Active			
3:27	Some activity			
3:53	Motionless	Sea-water added	3	1 hour 40 min.
3:57	Activation			
4:50	Motionless	Sea-water added	4	57 min.
4:52	Some activity			
5:12	Active			
5:51	Motionless	Sea-water added	5	61 min.
5:55	Some activity			
6:25	Active			
7:03	Motionless	Sea-water added	6	72 min.
7:05	Some activity			
7:47	Motionless	Sea-water added	7	44 min.
7:51	A little activity			
8:22	Motionless	Sea-water added	8	35 min.
8:30	No further activation			

clean dry glass needle, there was no activation, and even examinations with a magnification of 950 × failed to show any activity whatsoever. Dilution of dry spermatozoa from the same testis tubules with ordinary sea-water gave perfectly typical activation.

Two preparations with the supernatant fluid were made permanent with the edges of the cover glass sealed with petroleum jelly, one at 12:35 P.M., and one at 1:55 P.M. The spermatozoa in the former were still motionless at 2:21 P.M. but when fresh sea-water was added at that time, the spermatozoa became intensely active by 2:25 P.M. In the latter, the spermatozoa remained motionless until 7:30 P.M., but when fresh sea-water was added at that time, became intensely active by 7:37 P.M. *The residual spermatozoa after centrifuging showed normal activation in all cases when ordinary sea-water was added.*

This experiment definitely indicated that the suspensory fluid of dry spermatozoa presents a condition which serves to prevent fresh dry sperm from becoming active. This condition occurs also, in the supernatant fluid, when 3″ of dry sperm is centrifuged for 30 minutes at 2,500 revolutions per minute through $1\frac{1}{2}''$ of fresh sea-water. If the supernatant fluid from such centrifuging be removed and replaced with fresh sea-water, and the spermatozoa of the first centrifuging be centrifuged through this sea-water for 45 minutes at 2,500 revolutions per minute, the supernatant fluid from this centrifuging, too, will prevent the activation of dry sperm.

All gradations occur. All supernatant fluid obtained by any system of centrifuging dry sperm will prevent activation of fresh sperm. When more dry sperm than sea-water is present, one passage of the spermatozoa through the sea-water is sufficient to render the supernatant fluid capable of preventing activation of dry sperm. When, however, more sea-water than dry sperm is present, several washings, the number depending upon the condition of the spermatozoa and the relative proportions of sperm and sea-water, are necessary, and there are several experiments which demonstrate the relationship between the relative amount of dry sperm and sea-water present to the amount of centrifuging necessary to cause the supernatant fluid to prevent activity in dry sperm. Thus, in one typical experiment, dry sperm to sea-water in the proportions of $\frac{3}{8}'' : 3\frac{1}{4}''$, $1\frac{3}{16}'' : 2\frac{3}{4}''$, $2\frac{3}{8}'' : 2\frac{1}{2}''$, and $1\frac{7}{8}'' : 1\frac{1}{2}''$ were each washed five times, being centrifuged approximately 10 minutes at 3,550 revolutions per minute for each washing. The supernatant fluid from the $\frac{3}{8}'' : 3\frac{1}{4}''$ proportion did not stop dry sperm, whereas the supernatant fluid from each of the other proportions stopped dry sperm completely. In another typical experiment, the proportion of dry sperm to sea-water was made the same in all four tubes, namely $\frac{1}{2}''$: $3''$, and a tube removed after 5, 10, 15, and 20 washings of approximately four minutes at 3,250 revolutions per minute for each washing. The supernatant fluid after five washings did not stop dry sperm, the fluid obtained after 10 washings allowed "just a little activity" in dry sperm, while the supernatant fluid obtained after 15 and 20 washings completely prevented activity in dry sperm.

In order to make certain that the occurrence of the condition in the supernatant fluid was developed by the centrifuging, an exactly similar proportion of dry sperm and sea-water was made and thoroughly mixed at the same time that the other tubes were prepared. This suspension of spermatozoa was kept in a tube until the centrifuging of the other tubes had been completed. The sperm in this control tube were then separated from the suspensory fluid by means of one centrifuging at 3,250 revolutions per minute for 8 minutes, and the resulting supernatant fluid tested. It was found to be utterly incapable of preventing or reducing activity in dry sperm to any extent that could be optically determined.

These experiments indicate that some substance or condition develops or appears in suspensions of spermatozoa which has the property of preventing activity in the spermatozoa of that or of a fresh suspension of dry sperm. This substance or condition is rendered ineffective, or is reduced to sub-threshold concentration or intensity by the addition of fresh sea-water, but its concentration or intensity may be definitely and markedly increased by washing the spermatozoa through the suspensory fluid by means of the centrifuge.

Other Properties of Supernatant Sperm Fluid

When a drop of supernatant fluid which will definitely prevent activity in dry sperm is thoroughly mixed, by means of a clean, dry glass needle, with one drop of an egg-sea-water which will produce definite and clear-cut agglutination clumps with dry sperm and this mixture, in the form of a drop on a clean, dry glass slide, is brought into contact with a drop of dry sperm from the same tubules, and the whole process watched carefully with the microscope, the spermatozoa will be seen to become active in a manner exactly similar to that which occurs when a dilution is made with ordinary sea-water, but no slightest sign of any form or degree of agglutination whatsoever occurs. This test was repeated several times with many different preparations, and with many different lots of gametes, but always with the same result. In all cases when the supernatant sperm fluid would prevent activity in dry sperm, it would neutralize or destroy in some way the agglutinating power of egg-sea-water, but reciprocally, its power to prevent activity in dry spermatozoa was similarly destroyed, and the mixture of supernatant fluid plus egg-sea-water served to activate spermatozoa in a manner exactly similar to that of ordinary sea-water.

This phenomenon was even more vividly shown when a small drop of the supernatant sperm fluid was added, with a fine capillary pipette, to the middle of the field where the agglutinated clumps, produced by the addition of egg-sea-water to dry sperm, could everywhere be seen, and the whole process watched continuously under the microscope. The response was almost instantaneous. The agglutination clumps everywhere within the area of the added drop *immediately* dispersed, while outside the boundary of the drop and on all sides, clumps persisted in a perfectly typical and unaffected arrangement. Within the drop, the spermatozoa instantly became inactive, while along the boundary of the drop, some activity and motion could be seen.

The phenomenon, too, was readily demonstrated by making preparations on one slide which could be observed successively and sequentially, and directly compared, and which would consist of dry sperm mixed with (1) ordinary sea-water, (2) egg-sea-water, (3) supernatant sperm fluid, and (4) one drop egg-sea-water plus one drop supernatant sperm fluid. (1) and (4) showed typical activation, but no agglutination, (2) showed distinct and definite agglutination, while the spermatozoa in (3) were utterly inactive.

There seems to be a parallelism between ability to prevent activity of dry sperm and ability to neutralize egg-sea-water. All supernatant fluid that would prevent activity in dry sperm would also neutralize the agglutinating power of egg-sea-water. When the supernatant fluid allowed "just a little activity," the mixture of supernatant sperm fluid and egg-sea-water allowed "slight and evanescent tendencies" towards the formation of agglutination clumps, while finally, the supernatant fluid obtained from a sperm suspension that had been diluted for 2¾ hours, but that had not been washed by centrifuging, would not prevent activity of dry sperm, and would not prevent egg-sea-water from causing agglutination. These observations seem to indicate that the activity-preventing and the egg-sea-water neutralizing properties of supernatant sperm fluid are due, either to the same substance or condition, or to two or more substances which, however, arise or occur together in the supernatant fluid under all the treatments where the effects have been observed.

Discussion

Attempts to extract such substances from the spermatozoön as would have a relationship to the fertilization reaction have been made by Sampson (1926), Hibbard (1928), Wintrebert (1929, 1930*a*, 1933), Einsele (1930), and Parat (1933*a*). Sampson was unable to obtain any substances in sperm filtrates and dialysates which would activate "fertilizin" in the egg-sea-water so as to make it an efficient parthenogenetic agent, or would combine with the agglutinating substance in egg-sea-water so as to destroy its power to agglutinate fresh sperm suspensions, though she did obtain substances which would initiate development of mature ova of the same species. Hibbard and Wintrebert found that solutions of macerated spermatozoa would digest egg membranes, while Einsele and Parat found that filtered ether dialyzates and extracts of entire testes would, when injected into the egg with a micropipette, give an activation of the eggs in 60 per cent of the cases. Parat found that the development was much more regular, and that many more of the gastrulae would form larvae when the eggs were activated parthenogenetically by means of the introduction of the acrosome of spermatozoa removed and injected by means of microdissection needles and pipettes. Both Parat and Einsele have obtained evidence that the substance concerned in such parthenogenetic activations is a proteolytic enzyme.[3] Histo-anatomical studies of spermatozoa have been made by Bowen (1924), Popa (1927), Wintrebert (1930*b*), Parat (1928, 1933*b*) and others, and some of these studies indicate the presence of substances of secretory origin in the acrosomal region of spermatozoa.

Since egg-sea-water, reciprocally, will neutralize or destroy the activity-preventing or inhibiting substance of a sperm suspension, the activating property of egg-sea-water, as described by Lillie (1913), hereby receives an explanation. The power of the substances in the supernatant sperm fluid to neutralize the agglutinating power of egg-sea-water also conforms very readily with the fertilizin theory of Lillie (1919), and with the observations of Lillie (1919) and others with *Arbacia punctulata* that it was not possible at any time to regain agglutinating substances that had once been used in order to cause an agglutination of spermatozoa.

SUMMARY

1. Dry sperm of *Echinometra subangularis* is intensely active immediately after its removal from the testes tubules, but this motion

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[3] Recently (1939), Frank has described a sperm extract of *Arbacia punctulata* spermatozoa, obtained by heating the sperm suspension, which has the property of neutralizing the fertilizin of egg-sea-water, and which has an agglutinating effect on the cilia of *Arbacia* plutei. It is possible that the inactivating effect of the supernatant fluid of *Echinometra* spermatozoa is due to a similar action upon the tails of the sperm.

lasts for but from 50 to 120 seconds, when all activity ceases. When, however, a drop of sea-water is brought in contact with such a drop of dry sperm, the spermatozoa again become active, and this activity continues for from three to four hours. The addition of another drop of sea-water will cause the spermatozoa again to become active, and this activity continues with gradually decreasing intensity for about one and one-half hours. Such spermatozoa can again be reactivated, but the length of time during which they remain active progressively decreases, until finally, no further activation is obtained.

2. This inactivation of sperm suspensions with time is caused, probably, by the accumulation of a substance with time which has, as its characteristic identificatory property, the prevention of spermatozoa from activity. It occurs in the supernatant fluid obtained from centrifuging dry sperm, and in the supernatant fluid obtained by washing dry sperm through ordinary sea-water several times with the centrifuge, but the suspensory fluid of diluted sperm suspensions through which the spermatozoa have not been washed does not contain the substance in detectable amounts.

3. Supernatant fluid which contains this substance also, in all cases, has the property of neutralizing the agglutinating power of egg-sea-water, and there seems to be a parallelism between ability to prevent activity of dry sperm and ability to neutralize egg-sea-water.

4. These observations indicate that the activity-preventing and the egg-sea-water neutralizing properties of supernatant fluid are due, either to the same substance, or to two substances which, however, occur together under all the treatments where the effects have been observed.

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THE "AGGLUTINATION" PHENOMENON WITH SPERMATOZOA OF CHITON TUBERCULATUS

WALTER E. SOUTHWICK

(*From the Bermuda Biological Station for Research*[1])

INTRODUCTION

It has been shown by Crozier (1922) that when sperm, diffusing from a male individual of *Chiton tuberculatus* during the month of May (fully a month before ripe eggs are seen), was taken up between the ctenidia of a female, it issued from the posterior ends of the ctenidial channels principally in the form of "numerous agglutinated masses of active sperms" which persisted in sea-water for at least one half-hour. He found similar "agglutination" when sperm had passed through the ctenidial channels of males, and when it had been added (1) to ovarian extracts from mature eggs in sea-water, or (2) to sea-water into which ripe eggs had been shaken from an ovary and allowed to stand for half an hour. He considered these conditions to indicate that "mere evidence of sperm agglutination (cluster formation) may well have no bearing on the fertilization reaction." This conclusion is in distinct conflict with that formulated by Lillie (1919) on the basis of his observations on *Arbacia punctulata* and *Nereis limbata*.

The observations of Crozier (1922) have been extended during a series of observations made during the summers of 1933 and 1938 at The Bermuda Biological Station for Research.

OBSERVATIONS

Dry spermatozoa[2] from a mature male *Chiton tuberculatus* are homogeneously motile. When such dry sperm is introduced into the mantle cavity either of a male, or of a female which does not shed eggs, in a way such that it is caught up in the ctenidial current and carried

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[2] When a drop of such sperm is brought into contact with a drop of mature eggs on a glass slide, the egg contents, within about one minute, shrink visibly so that a clear area is produced around the egg and between the egg and the chorion. When such eggs are transferred to Syracuse watch crystals containing approximately 10 cc. sea-water, about 98 per cent of them will have cleaved, within approximately one and one-half hours, to form the two-cell stage.

through the gills to be discharged at the posterior end, this discharged sperm, if allowed to collect in the dish, or to rest undisturbed on a glass slide, can be seen to form macroscopically visible white masses in a short time, as Crozier observed. If, on the other hand, the discharged sperm is collected with a clean pipette, transferred to a glass slide, and examined immediately, a very interesting series of changes can be observed.

During the first few seconds, the spermatozoa swim about actively, freely, homogeneously. During the next few seconds, they come together and form small clumps of spermatozoa, *but the point of attachment is the tail*, while the heads remain perfectly free. Sometimes the group may consist of but ten or twelve spermatozoa, in which case the clumps resemble bouquets of flowers, tufts of grass, or even more appropriately, a handful of balloons waving in the breeze. Soon these clumps fuse into larger masses, either in such a way as to form complete spherical masses, as shown in Fig. 1, or much more frequently, to form strands of spermatozoa. These strands, at first, are slender and comparatively short, and are often but slightly branched, but soon they elongate, thicken and branch, as shown in Fig. 2. Soon these strands fuse with others, in a way such that within about three minutes extensive networks appear, as may be seen from Fig. 3. These networks soon become so large that they are macroscopically visible.

Careful observation of the mode of formation of these strands reveals that the process is quite comparable to a braiding of the tails of the spermatozoa. Always at the end of the strand, a tuft of spermatozoa with entirely free heads may be seen. These heads are continually waving back and forth, in and out, moved by the whipping motion of the spermatozoön tail. In a short time, the latitude of the motion becomes restricted, and the restriction progressively increases until finally only the head is free. It can then be seen, still actively waving, from the side of the strand. Often, however, the head is included in the braid.

As the sperm become more and more bound in the strand, other spermatozoa get caught and soon these are inescapably bound while others are caught. This continues until all of the free-swimming spermatozoa are bound, when the terminal tufts then persist for long periods of time, probably permanently. These tufts have been examined with especial care, and in every case, the sperm head has been found to be absolutely distinct and completely separate from its neighbors.

When, however, a drop of dry sperm on a glass slide is brought in contact with a drop of sea-water in a way such that the spermatozoa are carried into the sea-water by the resultant currents, it frequently happens that the sperm suspension is simply swept into the sea-water drop. Unlike the condition with most forms, the sperm mass tends to remain intact, and though it generally exhibits a slight increase in homogeneous motility, it shows no slightest sign of the clumping phenomenon. Occasionally, however, under such conditions, tufts of spermatozoa may form along the contacting edge, as may be seen in Fig. 4.

On the other hand, when the two drops are fused in a way such that the spermatozoa are distributed widely and rapidly into the sea-water, *all* stages that were observed with the sperm that had passed through the ctenidial channels could be seen repeated under such conditions. The phenomenon was first noted when the sperm drop was brought into contact, in the usual way, with a drop of sea-water to which a little ether had been added. The markedly reduced surface tension produced violent currents which served rapidly to carry the sperm to all parts of the drop. The ether evaporated rapidly, and by so doing probably produced still more currents, but did not appear to affect the motility of the sperm in any way. Later it was possible to repeat exactly the same series of changes with non-etherized, normal sea-water by the proper regulation of the relative sizes of the drops. When the two drops are fused with the aid of a clean glass needle in a way such that the sperm suspension is spread widely throughout the drop of sea-water, the typical strands, as shown in Fig. 2, form immediately and everywhere, and these soon anastomose to form the extensive net-works shown in Fig. 3. In these nets, the sperm heads frequently project into the interstices of the net and there continue to wave actively for a long period of time. Sometimes the network formation may be so extensive that distinct membrane-like structures are produced, which readily curl up and may readily be caused to wave in a manner typical of any such membrane, if the slide be moved or shaken gently under the microscope.

When a drop of dry sperm is introduced suddenly into 8 or 10 cc. of sea-water in a small beaker, the sperm mass may be seen to drop to the bottom of the beaker in the form of a much-folded membrane, resembling in every respect a piece of silk allowed to fall lightly on a table. Microscopic examination of this mass shows that it is composed of a still intact mass of homogeneously distributed spermatozoa. Though this membrane of spermatozoa is very delicate, it is possible to lift it, *as a membrane*, with a fine glass needle, and to fold it back upon itself, or to roll it into a markedly more compact mass. Tufts, in due time, appear along the edges of the mass, and circular clumps may

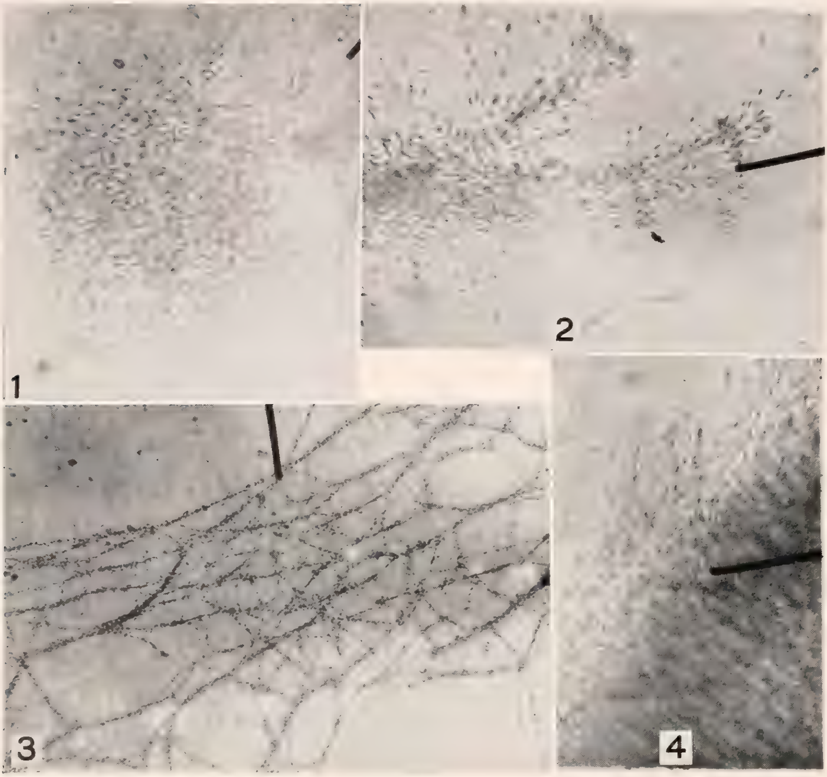

PLATE I

Fig. 1. The structure of the spherical masses that are formed by the spermatozoa of *Chiton tuberculatus* after dilution with sea-water, or after passage through the ctenidial chambers of a male or immature female. Sometimes the masses formed are more compact, but the structure, with the tails bound and the heads free, is the same in all cases. × 440.

Fig. 2. The elongating, thickened, branching strands that form when the spermatozoa of *Chiton tuberculatus* is passed through the ctenidial chambers of a male or immature female, or is diluted with sea-water. × 440.

Fig. 3. The nature of the network that forms by the fusion of the strands produced by the spermatozoa of *Chiton tuberculatus* when diluted with sea-water, or passed through the ctenidial chambers of a male or immature female. × 100.

Fig. 4. Photomicrograph to show the retention of mass integrity, with occasional formation of tufts along the contacting edge, when a drop of dry spermatozoa of *Chiton tuberculatus* is gently brought into contact with a drop of sea-water. × 440.

appear separated from, but near the margins of, the membrane. Rarely, strands may also appear. When, however, the original sperm mass becomes spread very thin, widespread formation of the network usually results.

If, however, some of the sperm be separated from the main mass, either by means of a glass needle, or by mechanical agitation, typical strands and networks are immediately formed. Any mechanical disturbance of the drop of dry sperm in order to obtain a uniform, homogeneous, diluted sperm suspension results only in a complete transformation of the sperm mass into strands, and a microscopic examination of sperm suspensions of one drop of dry sperm to 5, 10, 15, 25, 50, 100, and 150 cc. sea-water have all shown the presence of such strands, and the complete absence, in any case, of completely free swimming spermatozoa. These strands slowly settle to the bottom of the container, the rate depending upon their size, where they form into networks, and, if the concentration be sufficient, into more or less homogeneous membranes. These membranes can again be broken up into strands, and the process can be repeated several times. The strands in sea-water persist indefinitely, or as long as the spermatozoön tail is intact. In the more dilute suspensions, however, the strands remain quite uniformly distributed in the suspension for some time.

On the other hand, when extracts from the ovaries of ripe or spent females, obtained by cutting the ovary into small pieces and washing it thoroughly through cheese-cloth with about 25 cc. of sea-water, are used instead of sea-water, *no clumping in any form occurs.* Instead, the spermatozoa in the advancing edge of the drop of dry sperm move freely, and the sperm mass progresses steadily until the far side of the drop is reached. At no time do tufts, strands, or networks appear, and, furthermore, if spermatozoa be drawn from the mass by means of a glass needle, they simply disperse and soon merge imperceptibly with the other spermatozoa as the main mass advances upon and closes in about them.

Dilution of the ovarian extracts appears to lessen their effectiveness. A dilution of one drop of extract to ten drops of sea-water is often quite as effective as the full strength extract. A 1 : 25 dilution, however, prevents the formation of tufts and other similar structures, but does not remove the restriction to a free movement of the sperm through the drop of ovarian extract; while a 1 : 50 dilution allows the formation of tufts, clumps, strands, and networks just as though it were pure sea-water. These figures, however, are of relative value only, since there is a wide variation between the extracts from the different ovaries, as seen in the fact that one of those tested gave a perfectly typical clumping reaction with a 1 : 10 dilution. Clumps of spermatozoa formed by dilution of dry sperm with sea-water can be caused to disperse by the addition of ovarian extract.

When egg-sea-water, made by allowing the eggs of one female to stand in 25 cc. sea-water for half an hour, was used, exactly similar effects were obtained to those obtained with the ovarian extracts. Of course, dilution of this egg-sea-water reduced the effect, just as was the case with the ovarian extract. When, too, a drop of dry sperm was brought in contact with a drop of eggs and watched under the microscope, the sperm could be seen to move freely across the open spaces, and to gather about the eggs, but there was no sign whatsoever of a clumping reaction. These latter observations are in accord with, and to an extent provide an explanation of, the observations of Crozier (1922) that "during natural fecundation, however, no spermballs are formed. The thick, glutinous stream of spermatozoa passes under the girdle of the female, is somewhat diluted with sea-water by the tractive current, and emerges posteriorly in company with numerous large greenish eggs, about which, under the microscope, it can be seen that many sperms are gathered. But no real 'cluster formation' takes place."

Other substances that prevent clumping include the body juices of a mature male, body juices of a mature female, as was also noted by Crozier (1922), sublethal solutions of saponin in sea-water,[3] similar solutions of sodium taurocholate in sea-water, saturated and somewhat diluted solutions of trypsin in sea-water, and possibly by other substances. On the other hand, no prevention of clumping was obtained with acetone, ether, methyl alcohol, ethyl alcohol, adrenaline chloride (1 : 1000, 1 : 2000, or 1 : 5000), or carbon disulphide.

DISCUSSION

It is obvious that in *Chiton tuberculatus*, the clumping reaction is different in every way from the agglutination reaction which has been described by Lillie (1913) for *Arbacia punctulata*, by Loeb (1914) for *Strongylocentrotus purpuratus* and *S. franciscanus*, by Just (1919) for *Echinarachnius parma*, and later (1929) for *Paracentrotus lividus* and *Echinus microtuberculatus*, by Carter (1932) for *Echinus esculentus*, by Lillie (1913) for *Nereis limbata*, by Just (1915) for *Platynereis megalops*, and by other workers with other forms. With the agglutination reaction in these cases, the agglutination is between the heads of the spermatozoa, while the tails are apparently unaffected; the agglutination reaction is spontaneously reversible, cannot be repeated, and generally is produced only by substances secreted by eggs of the same species. With the clumping reaction in *Chiton tuberculatus*, on the other hand, the agglutination is between the tails while the heads are apparently utterly free and unaffected; the clumps, when once formed, persist indefinitely unless dispersed by means of the addition of certain substances, or by mechanical means. In the latter case, the clumps will reappear with an almost endless number of repetitions. The clumping forms as a perfectly natural and normal result of dilution with ordinary sea-water, and is, in addition, a phenomenon which can be prevented by means of body juices of the same form, egg and ovarian secretions and extracts, and certain lytic substances, such as saponin and sodium taurocholate.

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[3] The sources of the substances used for these tests were as follows:

Saponin: Eimer and Amend, New York, "A-61 Purified."
Sodium taurocholate: Eimer and Amend, New York, "A-61 Purified."
Trypsin: Eimer and Amend, New York, "A-61" "Pure."
Acetone: U. S. P., J. T. Baker Chemical Co., Lot No. 92237.
Adrenaline Chloride: Parke, Davis and Co., Detroit, Mich., U. S. A.
Ether, methyl alcohol, ethyl alcohol, and carbon disulphide: Usual laboratory supplies.

With *Chiton tuberculatus*, it is unlikely that the clumping reaction, first described by Crozier, and described in detail herein, has any *direct* relation to the fertilization reaction as such. Instead, it is probably a mechanism by means of which masses of dry sperm may be transferred, in an intact manner, from the male to the mature female. This mass of dry sperm, thus transferred in an essentially intact condition, comes in contact with substances in the ctenidial channels of the female which destroy the substance which causes the tails to stick together and thus form clumps. The spermatozoa thus become freed from each other and are then able, by their own individual and utterly independent movements, to activate the all-environing eggs. The reason for transferring the spermatozoa to the female in an *intact* mass, however, might possibly reside in the need to preserve substances which might be essential for the actual fertilization reaction, and which might be rapidly lost from the spermatozoön in less concentrated suspensions.

The fact that these clumps can be dispersed by means of the proteolytic enzyme, trypsin, and by the lytic substances saponin and sodium taurocholate indicates that the clumping reaction in this form rests, fundamentally, upon the presence, on the outside surface of the spermatozoön tails at the time they are liberated from the testes, of a substance which (1) is distinctly sticky in nature, and which (2) can be dissolved or destroyed by the above-mentioned substances. Since lytic substances, as suggested by Ponder (1930), might act by the destruction of the structure of proteins, a process which is also hastened by trypsin, it is possible that the substance on the tails of the spermatozoa of dry sperm suspensions might be a sticky protein of some sort. On the other hand, Fieser (1937) has suggested that the hemolytic effect, of saponin at least, might be produced by a combination with cholesterol or lecithin of the cell membrane in a way such as to render the membrane permeable to hemoglobin, and evidence in partial support of this suggestion has been provided by Ransom (1901) in that he has shown with certainty that a combination of saponin and cholesterol is possible and that treatment of a saponin solution with cholesterol destroys its hemolytic activity. Popa (1927) has obtained evidence that the tails of the spermatozoa of *Arbacia punctulata* and *Nereis limbata* are enveloped by a large amount of lipoid substance.

SUMMARY

1. When dry sperm of a mature male *Chiton tuberculatus* is introduced into the mantle cavity of a male or immature female and the discharged sperm is collected and examined immediately with a microscope, the spermatozoa will be seen to come together and form small clumps. *The point of attachment of these clumps is the tail*, while the heads remain perfectly free.

2. These clumps fuse readily to form either large spherical masses, or strands, which, in turn, soon fuse with other strands to form extensive networks. Such structures also form, readily and extensively, when the two drops are fused with the aid of a glass needle in a way such that the sperm suspension is distributed widely throughout the drop of sea water.

3. On the other hand, when extracts from the ovaries of ripe or spent females, egg-sea-water, body juices of a mature male, or of a mature female, or sublethal solutions of the lytic substances, saponin or sodium taurocholate, are used, *no clumping in any form occurs.* Instead, the spermatozoa in the advancing edge of the drop of dry sperm move freely, and the sperm mass progresses steadily until the far side of the drop of diluting fluid is reached.

4. These observations indicate that, in *Chiton tuberculatus*, the clumping reaction rests fundamentally upon the presence, on the outside surface of the spermatozoön tails, of a substance which (1) is distinctly sticky in nature, and which (2) can be dissolved or destroyed by certain substances.

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THE LUMINESCENCE OF A NEMERTEAN, EMPLECTONEMA KANDAI, KATO

SAKYO KANDA

(*From the Institute of Physical and Chemical Research, Hongo, Tokyo, Japan*)

Introduction

A great many species in the five phyla, Plathelminthes, Nemertea, Trochelminthes, Nemathelminthes and Chaetognatha, are closely allied to one another. Among these species, no luminous form has been previously recognized. I found, however, a number of luminous nemerteans, when I visited the Marine Biological Station of the Tohoku Imperial University at Asamusi, Aomori, Japan in the summer of 1936.

These nemerteans had coiled up on *Chelyosoma*, which were collected from the bottom of Aomori Bay between Natutomari and Aburame at a depth of about 35–40 meters, and were placed in the laboratory for study. They were identified by Koziro Kato (paper in preparation) as *Emplectonema kandai* sp. nov.

It is an extraordinary fact that among so large a number of species of the five phyla, only one is found to be luminous. *Emplectonema* is a genus of the nemerteans which is widely distributed in America, the Atlantic and Pacific Oceans, the Mediterranean Sea, the White Sea and Japan. It may be expected, therefore, that more luminous species of the same genus, at least, will be observed somewhere in the future. I made some experiments on *Emplectonema kandai* during the three summers of 1936–38 at the Station mentioned above. The results are given in the present paper.

I wish to express my sincere appreciation of the facilities afforded me there by Professors S. Hatai (1937) and S. Hozawa (1938), Directors of the Laboratory. I would also like to acknowledge my indebtedness to Messrs. N. Abe, K. Atoda and K. Kato, without whose aid this paper would not have been completed.

Material

As already stated, these luminous nemerteans coiled up on *Chelyosoma*. It is necessary, therefore, to collect the latter, which are dredged (by three fishermen) from the bottom of Aomori Bay, about

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[1] A preliminary note in Japanese was published in the *Rigakukai*, **35** (1937): 5–11.

15 km. off the Station. But the nemerteans are not abundant. Only six or seven individuals at best, or sometimes only one or two individuals, are obtained on about two hundred *Chelyosoma*, which are collected by the fishermen as the result of one day's work.

The nemerteans are reddish orange in color. They have many eyes. They vary in length, from 53–115 cm., and are about 0.5–0.7 mm. in diameter, when they are stretched. I found one individual 10 cm. long, but one so short is extremely rare. The female animal is readily distinguished during the summer season because it is full of eggs or enlarged gonads, but I could not distinguish the males. The animals coil up on the wall of a large vat of running sea water or on the bottom, attached by the slime which is abundantly secreted from the surface of its body (Fig. 1). They remain there quietly for two months or more, if they are not disturbed.

FIG. 1. The living and coiled whole *Emplectonema kandai* about 115 cm. long. About natural size. (Photographed by N. Abe at my request.)

The animals flash brilliantly only on stimulation. The stimulus may be mechanical, chemical, thermal or electrical. The light may appear on all parts of the body, but it disappears in one or two seconds. It is whitish green in color.

Mechanical Stimuli

The animal flashes when a glass rod or a finger is gently touched to the surface or surfaces of the coiled body. The light does not spread very far from the place or places of the contact, and lasts for only one or two seconds. Its intensity varies, depending on the strength of the contact. I thought at first that some luminous material was thrown into the sea water mixed in a slime discharged from the surface of the animal body, but this observation turned out to be incorrect.

If the coiled animal is strongly rubbed between the fingers, a brilliant light appears, but it is not observed that any luminous material comes off which adheres to the fingers. If the animal is suddenly extended, without being broken, between two hands, the head in one hand and the tail in the other, the brilliant light also appears through the whole surface of the long body except the tip of the head. This luminescence is a most beautiful sight.

Chemical and Osmotic Stimuli

If the sea water containing the nemertean is acidified with a very dilute HCl or acetic acid, the animal gives a bright light. The acid should not be too strong, or it will kill the animal too quickly. The dead or dying nemerteans produce light continuously, until all the luminous material is probably exhausted. The addition of dilute NaOH or NH_4OH to the sea water produces the same effect, although it precipitates the Ca and Mg of the sea water.

The best way to test the luminescence of the animal is to add dilute H_2O_2 to the sea water. This action is not injurious and is reversible.

If 1 to 2 cc. each of $\frac{M}{2}$ NaCl, $\frac{M}{2}$ KCl, $\frac{M}{2}$ NH_4Cl, $\frac{M}{2}$ $MgCl_2$, $\frac{M}{2}$ $MgSO_4$, $\frac{M}{2}$ Na_2SO_4, or $\frac{M}{2}$ $(NH_4)_2SO_4$ solution are added to 100 cc. of sea water which contains a nemertean, no luminescence is observed. In 0.5 cc. $\frac{M}{2}$ $CaCl_2$ plus 100 cc. sea water, however, the animal begins to flash occasionally after about 10 minutes. The intervals of its flashing become quite regular after about 20 minutes, resembling those of a firefly. Besides these flashes, there is a very faint and continuous light in other parts of the body. If this treated animal is removed to normal sea water after about 40 minutes, it lives normally. In 1 or 2 cc. $\frac{M}{2}$ $CaCl_2$ plus 100 cc. sea water, the intervals of the flashing are very slow and somewhat irregular.

In pure $\frac{M}{2}$ KCl, $\frac{M}{2}$ $CaCl_2$ or $\frac{M}{2}$ Na_2SO_4 solution, practically isotonic with sea water, the animal gives a bright light. But if the animal is kept too long in the solution, it will be killed. A mass of slime is secreted into each solution, but no luminous material is observed in it. In pure $\frac{M}{2}$ $MgSO_4$ solution, the animal flashes after about 8 minutes. In pure $\frac{M}{2}$ NH_4Cl solution, it gives a faint light after about 20 minutes. It would seem that K, Ca, Na, Mg, or NH_4 ions cause the luminescence of the animal.

In pure $\frac{M}{2}$ NaCl or $\frac{M}{2}$ $MgCl_2$ solution, isotonic with sea water, the animal gives no light. In pure $\frac{M}{2}$ $(NH_4)_2SO_4$ solution, also, no luminescence appears. In these cases, no cation, Na, Mg or NH_4, seems to cause any light whatever. It is a little difficult to decide from these experiments whether cation or anion stimulates the animal to become luminous since no luminescence is observed in NaCl, $MgCl_2$ or $(NH_4)_2SO_4$ solution, whereas some luminescence occurs in Na_2SO_4, $MgSO_4$ or NH_4Cl solution.

If crystals of NaCl, KCl, NH_4Cl, $CaCl_2$, $MgCl_2$, $MgSO_4$, Na_2SO_4, or $(NH_4)_2SO_4$ are added to 20 cc. of sea water, which contains the nemertean, a brilliant light is always observed. If a large amount of salt is used, the light is continuous and fades gradually, due to the death of the animal. On addition of fresh water or distilled water to the sea water, a bright light appears also. Saponin acts in the same way. The increase or decrease of osmotic pressure plays, of course, a distinct rôle in each case.

Temperature and Electrical Stimuli

If the sea water at 20° C., which contains the animal, is heated to 32–33° C., or is cooled to about 1° C., the animal produces light. With induced currents, the animal also gives light.

Luciferin and Luciferase

If the animals are placed on a heavy blotting paper, they give a bright light immediately. When they are dried over P_2O_5, light is still observed during the drying and the dried, dead animals give light when again moistened with water. When these moistened ones are dried again over P_2O_5, however, they produce no more light on being moistened again with water. The animal is slender and all parts of the body are covered by a simple, thin epithelium, where the luminous cells are located, as are the cells of other luminous animals. Evidently the luminous cells of the nemertean are not large, as the cross-section of the animal indicates (Fig. 2). This may explain why the amount of luminous material secreted by the cells is comparatively small.

The existence of luciferin and luciferase cannot be demonstrated in the usual way with either the fresh or the dried animals, which are ground with sand in a mortar and are extracted with hot or cold water. Methyl or ethyl alcohol extracts of the fresh and dried nemerteans also give no light with cold or hot water extracts. The cold water extract of the nemertean gives no light with *Cypridina luciferin*, nor does the hot water extract of the nemertean give light with *Cypridina luciferase*.

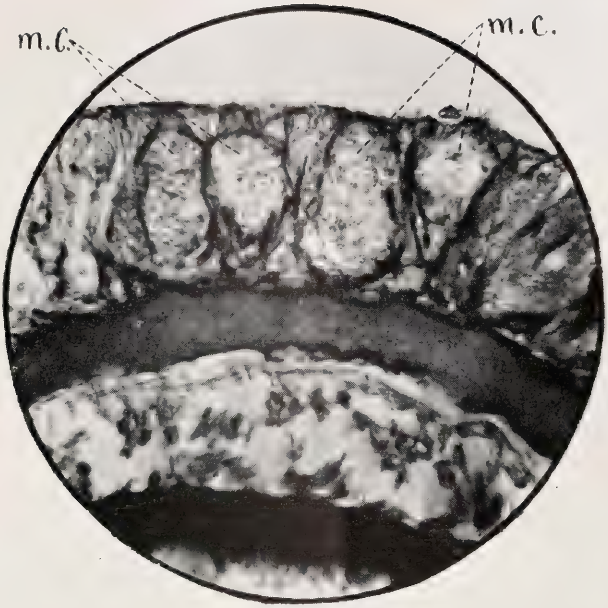
Potassium Cyanide

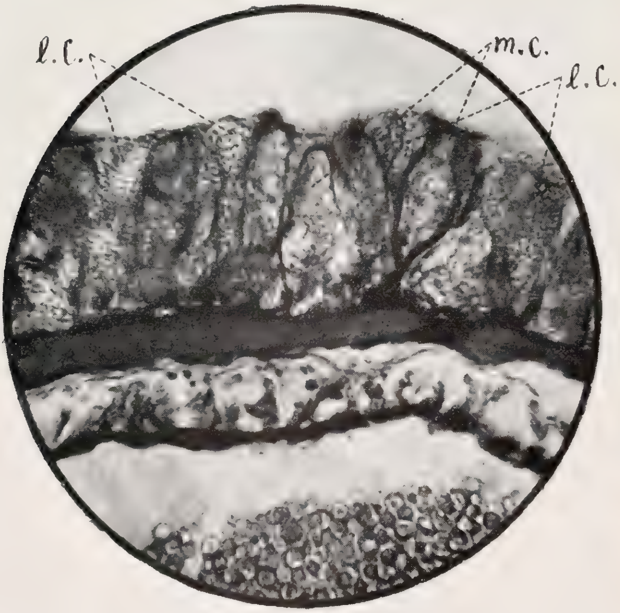
Since the luciferin-luciferase reaction cannot be demonstrated, the question may be asked: Is not the luminescence of the nemertean due to the symbiosis of luminous bacteria? Pierantoni (1918) holds that the light of all animals is due to symbiotic luminous bacteria. I did not attempt to raise luminous bacteria from the nemertean on an artificial culture medium, but studied the effect of KCN. According to Harvey (1921), the light of marine luminous bacteria disappears in 4 minutes, if they are treated with $\frac{M}{20}$ KCN solution, namely 0.325 per cent solution, and in 6 minutes, if treated with $\frac{M}{40}$ KCN or 0.162 per cent solution. He also shows that the light of an emulsion of the luminous organ of a fish, *Photoblepharon*, which is suspected to be symbiotic, disappears in about 20 minutes, if it is treated with 0.25 per cent KCN solution, and in about 30 minutes, if treated with 0.125 per cent KCN solution. I have found that the nemertean gives light immediately and that the light continues for about 110 minutes, if 1 cc. of the aqueous solution of $\frac{M}{2}$ KCN is added to 10 cc. of sea water which contains the animal. The animal begins to give light after 50 minutes and continues for about 140 minutes, if 0.5 cc. of $\frac{M}{2}$ KCN solution is added to 10 cc. of sea water. In both cases, the animal dies, not from dilution of sea water, but from KCN, since the worm can live indefinitely without evident injury in 100 cc. of sea water plus 20 cc. of distilled water.

These facts indicate that KCN, which inhibits most cell oxidations instantly, has very little effect on the luminescence of the nemertean. They would seem, also, to show that the light of the animal is not of bacterial origin. The failure to prove the presence of luciferin and luciferase in the animal does not necessarily indicate the symbiosis of luminous bacteria. On the contrary, I believe that luminescence in this nemertean arises from a chemical luminous material secreted in its luminous cells.

FIG. 2. Microscopic photograph of portion of a transverse section of the body surface of *Emplectonema kandai*, showing mainly the mucin-secreting cells (*m. c.*). About × 500. (Section prepared by K. Atoda at my request.)

FIG. 3. Microscopic photograph of portion of a transverse section of the body surface of *Emplectonema kandai*, showing some light-producing cells (*l. c.*) and mucin-secreting cells (*m. c.*). About × 500. (Section prepared by K. Atoda at my request.)





Figs. 2 and 3.

Histology

I have studied the transverse and longitudinal sections of this worm, which had been kindly prepared by K. Atoda and K. Kato. The epithelium of the worm is very simple, though it is comparatively wide. In general, there appear in the epithelium, two kinds of glandular cells which stain with Delafied's haemotoxylin and eosin. Those which stain blue with haemotoxylin are large and open through the cuticle of the epithelium (Fig. 2). They are apparently the mucin-secreting cells. In some preparations, however, a great many cells are almost devoid of slime, which was probably discharged while the worm was being narcotized with menthol.

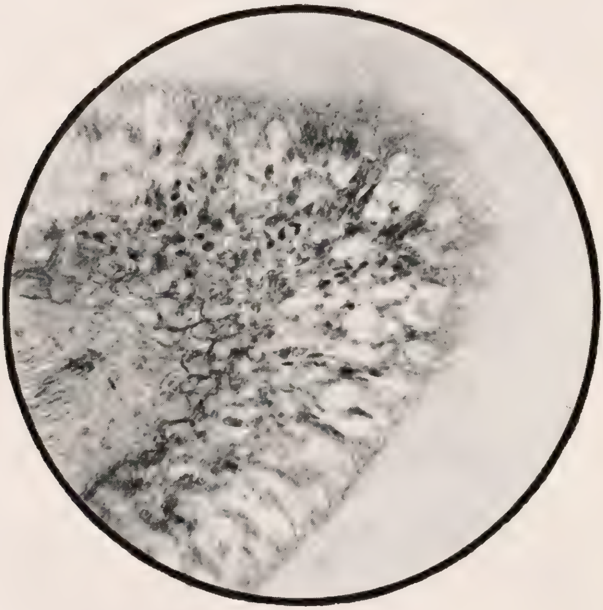


FIG. 4. Microscopic photograph of portion of a longitudinal section of the head-tip surface of *Emplectonema kandai*, where no light cells show. About × 500. (Section prepared by K. Kato at my request.)

The cells staining with eosin appear to consist of two types, although this is not always evident. Those of one type, which stain red with eosin, though not very deeply, show a small nucleus at the base, are elongate and open through the cuticle. They are filled with granules. These cells are most common throughout all preparations studied. The cells of the other type are especially evident when Mallory's stain is used. They stain deeply with eosin. Under a high power of the microscope they are seen to contain fine granules and in some cells their content is homogeneous. I assume that these cells are merely the young, unripe ones of the second type.

I believe that all the cells which stain in eosin are the light cells of the worm (Fig. 3). It is interesting to note that the tip of the head of the worm, where no light appears, as already stated, shows none of the eosinophil cells at all (Fig. 4.) In the head or anterior part farther from the tip, however, there appear some cells, which stain deeply with eosin and also contain the granules typical of the light cells. The number of such cells increases gradually towards the middle of the worm.

As I have already stated, the luminous material of the worm has not been observed to separate from the cuticle. But this does not mean that the glandular structure of the ducts has no opening or pore in the cuticle. On the contrary, all the ducts appear to have openings, as the sections show. The luminous secretion should be very small, however, as the light cells are also small, and the light production may take place at the instant of discharge, or the light-giving action may take place in the cells before the substances reach the openings of the ducts in the cuticle.

This work was aided by a grant from the Foundation for the Promotion of Scientific and Industrial Research of Japan.

Summary

The luminescence of a marine nemertean worm, *Emplectonema kandai*, living on *Chelyosoma*, is described. Light appears from the whole of the body, except a small region at the head end, in response to mechanical, chemical, thermal or osmotic stimulation. The effect of salts has been studied.

The photogenic cells are in the epithelium, stain with eosin, and appear to have openings in the cuticle, but no extracellular luminous secretion could be demonstrated. Histological sections are figured.

Luciferin and luciferase could not be demonstrated, but since KCN does not inhibit luminescence, the origin of the light is thought to be the gland cells of the worm and not symbiotic bacteria.

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ABSENCE OF THE EPITHELIAL HYPOPHYSIS IN A FETAL DOGFISH ASSOCIATED WITH ABNORMALITIES OF THE HEAD AND OF PIGMENTATION

DON WAYNE FAWCETT

(*From the Department of Anatomy, Harvard Medical School*)

The specimen herein described is an albino fetus of the spiny dogfish (*Squalus acanthias*) which presents malformations of the head including cyclopia and astomia. Associated with these is the very rare anomaly—absence of the epithelial hypophysis. It is well known that there are abnormalities of the pituitary in human anencephalic fetuses, but these involve absence of the neural lobe. Covell (1927), in making a quantitative study of such abnormal human fetuses, reviewed the literature and reported personal observations comprising in all nearly a hundred cases. He concluded that an hypophysis is always present, although the lobus nervosus is lacking in the majority of specimens. The pars anterior he found constitutes most of the gland volume and in some cases the total volume. He mentions no case of absence of the anterior lobe. The only instance hitherto described of spontaneously occurring absence of the anterior pituitary, in any animal, is the case reported by Evelyn Holt (1921) of absence of the pars buccalis in a 40-mm. pig.

The study of the present specimen of an elasmobranch fetus has been undertaken not only on account of its rarity but because of the evidence of interdependence in development of the separate lobes of the pituitary gland and the effect of absence of the oral components of the gland on pigmentation.

Material and Methods

The fetus was discovered among a great number of normal *Squalus* pups, at the David Richardson Laboratory, Bailey Island, Maine. Because its abnormalities escaped notice when it was removed from the uterus, normal littermates are not available for study as controls. Two normal pups, from other uteri, but of a comparable stage of development, were selected, instead, as controls. The three fetuses were fixed in 7 per cent formalin. The heads were subsequently imbedded in celloidin, cut serially in $35\,\mu$ sections and stained with haematoxylin and eosin.

Description

The specimen is silvery white with the exception of the tip of the dorsal fin and the tip of the tail where in each case a small area is deep grey, approaching in intensity the color of a normal pup. The otherwise translucent skin is faintly clouded with light grey over the dorsum of the body, suggesting that the albinism is not due to complete absence of pigmentation. Aside from this general albinism, the obvious external deviations from the normal are limited to the region rostral to the first gill slits; the rest of the body is of normal configuration. The eyes give the appearance of having been drawn from their usual lateral position ventrally and medially to the midline where they are fused into a single dumbbell-shaped eye with two discrete lenses. There are no external nares; only a midventral prominence indicates where the olfactory bulbs have coalesced. The mouth is represented by a dimple-like depression and a narrow fold formed by a shallow, rostrally directed invagination of the integument (Fig. 1). In addition to these abnormalities of the head, it is noted that the yolk sac is exceptionally large as compared to that of normal fetuses.

Examination of the sections reveals radical departures from the normal structure of the cartilaginous cranium, a description of which requires frequent reference to the normal processes of development in order to understand and interpret them correctly. The base of the elasmobranch chondrocranium normally develops from two pairs of cartilaginous bars. The posterior or parachordal cartilages constitute the caudal part of the basis cranii. In the anterior or prechordal region, the cranial floor develops from paired trabeculae cranii whose rostral ends fuse in the midline to form the interorbital plate and the more anterior rostral plate. Between the diverging posterior ends of the trabeculae and the anterior edge of the basal plate is a median space, the fenestra hypophyseos, through which the hypophysis and carotid arteries gain entrance to the cranial cavity. The original hypophyseal connection with the oral cavity is gradually obliterated by centripetal growth of these cartilages. In the present malformed cyclopean fetus the abnormalities of the chondrocranium itself are limited almost entirely to the prechordal region. Cyclopean teratogenesis is generally believed to occur very early in embryonic development of the eyes and is attributed to a local arrest of growth ventrally, with fusion of the elements of the primary optic vesicles and normal growth of dorsal parts. It is commonly thought that the cyclopean eye obstructs the path of forward growth of the cranial trabeculae—and this appears to have occurred in the present specimen. As one can observe in Figs. 7 and 8, there is an amorphous horizontal plate

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of cartilage which overlaps the back of the posterior edge of the eye and extends caudally to the level of the spiracle. Even though this cartilage does not unite with the basal plate at any point, it seems probable that it represents a fusion of the trabeculae in a somewhat abortive attempt to grow forward past the obstructing eye. This cartilage does not approach the basal plate caudally, as might be expected, because of the interposition of jaw elements. The primordium of the mandibular arch normally takes the form of an inverted *U* at each corner of the mouth. In this specimen the dorsal parts of the head have unfolded normally, carrying lateralward the pterygoquadrate limb of the *U*-shaped cartilages while at the same time arrest of growth in the midventral line has caused the mandibular limb (Meckel's cartilages) to be crowded against the median basihyoid plate. Subsequently there appears to have been more or less fusion of these cartilages such that, at the level of Fig. 9, they constitute a single plate of cartilage in which the pterygoquadrate components are represented most laterally, Meckel's cartilages next, and the basihyoid cartilage in the middle. The homologies ascribed to the abnormal cartilaginous elements seem justified because the conspicuous adductor mandibuli muscle complex which normally surrounds the angle of the jaw between pterygoquadrate and mandibular elements (Fig. 9-*a*) is oriented with respect to the abnormal fused mass of cartilage precisely as would be expected if the above explanation were correct (Fig. 9).

The brain is for the most part normal save for minor readjustments in relation to the misplaced olfactory bulbs and retinae. In the normal dogfish the floor of the diencephalon bulges ventrocaudally forming the infundibulum which consists of two hollow oval lobes—the inferior lobes. A long tongue of glandular tissue consisting of the pars distalis and pars medialis of the pituitary extends anteriorly from the pars intermedia and may be seen lying in the groove between the inferior lobes (Fig. 5-*a*). At the level of the emergence of the oculomotor nerves the inferior lobes of the infundibulum are normally continuous with a thin-walled vascular outgrowth, the saccus vasculosus (Fig. 6-*a*), which is connected posteroventrally with the glandular intermediate lobe. At this point, in the normal animal, nervous tissue from the thickened floor of the saccus vasculosus is commingled with

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PLATE I

FIG. 1. Ventral view of the abnormal *Squalus acanthias* fetus (natural size). The yolk-sac has been removed.

FIG. 2. Ventral view of a normal dogfish fetus.

FIG. 3. Dorsal view of the abnormal albino dogfish fetus.

FIG. 4. Dorsal view of a normal dogfish fetus.

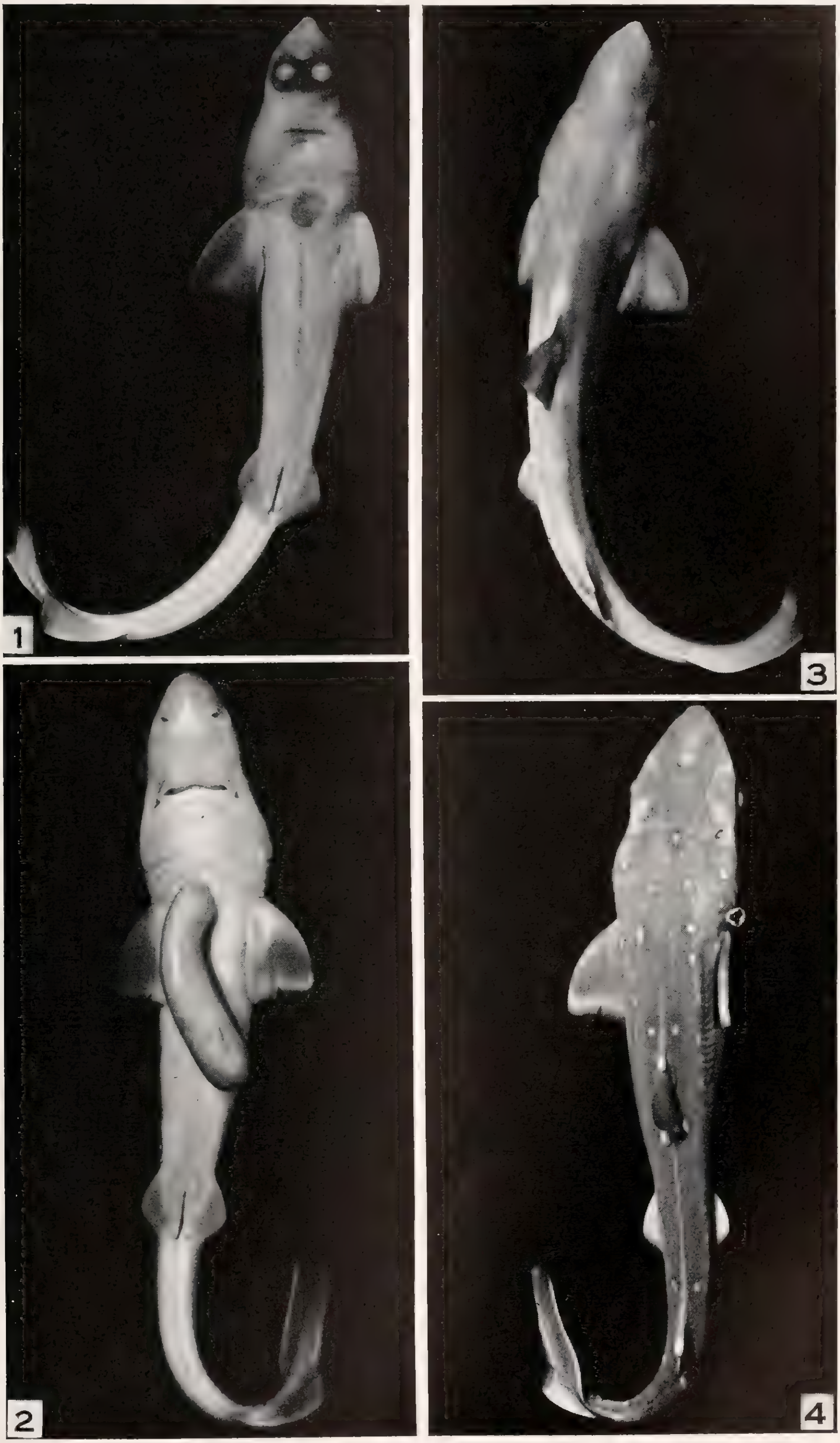
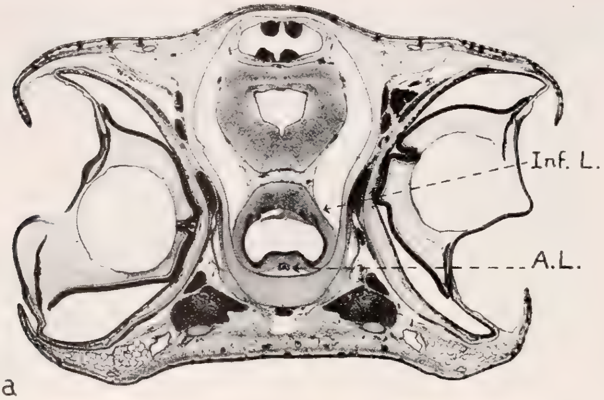


PLATE I

the glandular cords of the pars intermedia. The nervous tissue around this area of contact is usually thought to be the functional pars neuralis. The pars neuralis undergoes but little differentiation, the cells around



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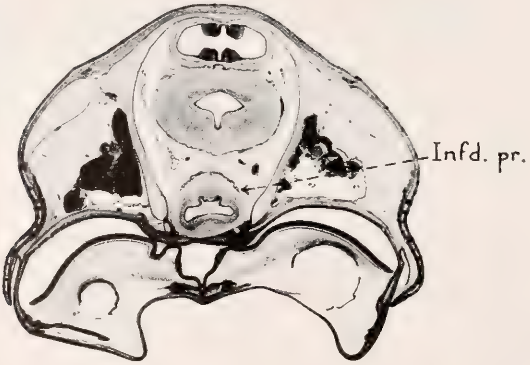

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PLATE I

FIG. 5-*a*. A section of the head of a normal fetus showing the pars distalis of the anterior lobe (*A.L.*), lying in the groove between the inferior lobes of the infundibulum (*Inf.L.*). Photomicrograph (hematoxylin and eosin; × 4).

FIG. 5. A corresponding section of the head of the anomalous fetus showing no anterior lobe and a deformed infundibular process (*Infd. pr.*) not supported beneath by an interorbital plate of cartilage. Photomicrograph; × 4.

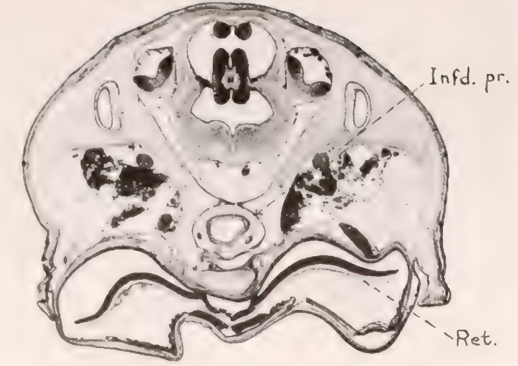
the infundibular lumen retaining much of the character of the embryonic ependymal layer (Butcher, 1936).

In the present abnormal specimen, on the contrary, the infundibulum extends posteriorly in a long conical process which, due to absence of the anterior part of the cranial floor, is for some distance not supported beneath by cartilage but traverses the groove between the two dorsal convexities of the malformed eye (Fig. 5). This attenuated infundibulum becomes gradually more slender as it passes out of the cranial cavity beneath the anterior edge of the basal plate. The basal plate dorsally, the pterygoquadrate and mandibular rudiments laterally, and a ventral plate of cartilage (possibly the basihyoid) are all partially fused about a tubular space in which the narrow infundibulum is lodged (Fig. 7). Caudal to this point these fused cartilages separate from the base of the chondrocranium providing much more space for the infundibular process (Fig. 8). In consequence of this, the infundibulum expands at its caudal tip into a bulbous enlargement which ends blindly at the level of the spiracle in contact with the blind end of the entodermal pharynx (Fig. 9). The infundibular recess which extends from the third ventricle into the infundibular stalk is nearly obliterated at its narrowest point but finally terminates in a conspicuous cavity within the terminal bulbous enlargement of the neurohypophysis. At no place does the infundibular process show any tendency to differentiate into a saccus vasculosus.

The most striking abnormality of the pituitary is the total absence of the epithelial portions of the gland. No trace can be found of the parts of the pituitary deriving from the embryonic stomodeum—pars distalis, pars medialis, pars intermedia, and pars ventralis.

Discussion

The pigmentary deficiencies, the absence of all buccal components of the pituitary, and the aberrant infundibular process exhibited by this anomalous fetus are of interest in connection with the findings of P. E. Smith in albino tadpoles produced by early ablation of the pars buccalis of the hypophysis. It was shown by him that atypically placed buccal epithelium would induce hypertrophy of adjacent nervous tissue whereas in complete absence of the buccal hypophysis, the neural lobe did not attain its normal size, shape, or histological development. Evelyn Holt's 40-mm. pig, while lacking entirely the oral portion of the hypophysis, is described as possessing a pars neuralis "normal in position, extent, and structure." This, she points out, is contradictory to Smith's findings but it is noteworthy that her specimen is from a relatively early stage of development. If the pig fetus had had an opportunity to continue its intrauterine life, the further growth of the pars neuralis might well have been retarded or modified.



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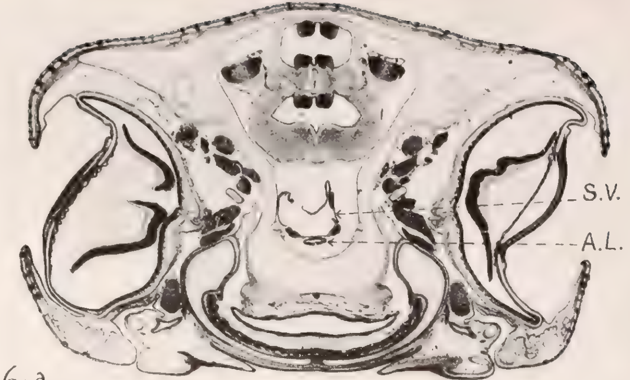

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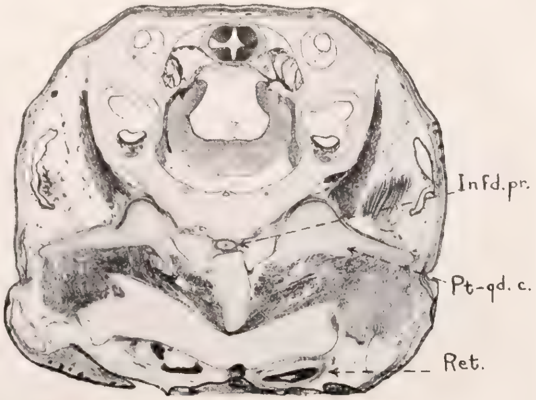

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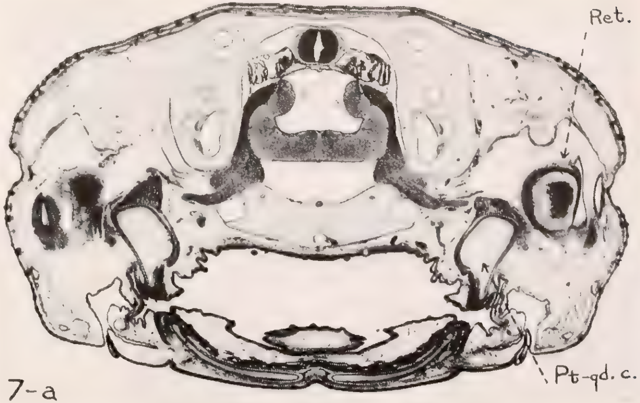


7-a

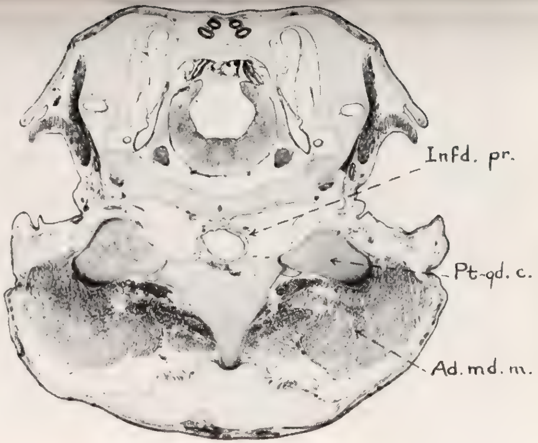
PLATE II

FIG. 6. Section of the head of the abnormal specimen showing the undifferentiated caudal extension of the infundibular process (*Infd. pr.*).

FIG. 6-*a*. Section at a corresponding level of the head of a normal control showing a saccus vasculosus (*S.V.*), and anterior lobe (*A.L.*) of the pituitary. Photomicrographs × 4.

FIG. 7. Photomicrograph of a section of the abnormal fetus at the level of the posterior edge of the retina (*Ret.*) showing the constricted region of the infundibular process (*Infd. pr.*) lodged between closely surrounding cartilages. Pterygoquadrate cartilage (*Pt-qd. c.*).

FIG. 7-*a*. Photomicrograph of a corresponding section of the normal control showing no part of the hypophysis present at



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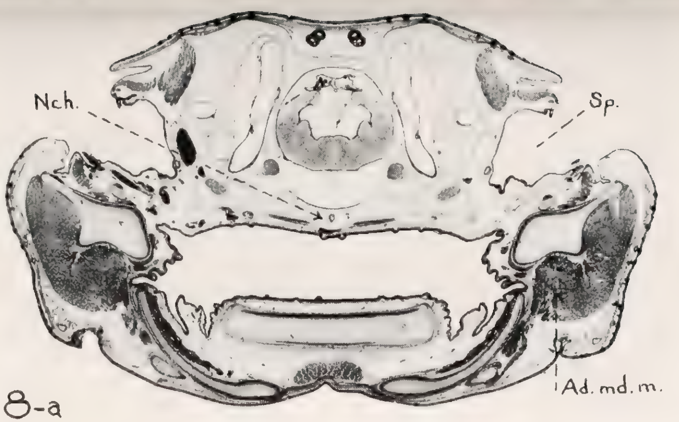

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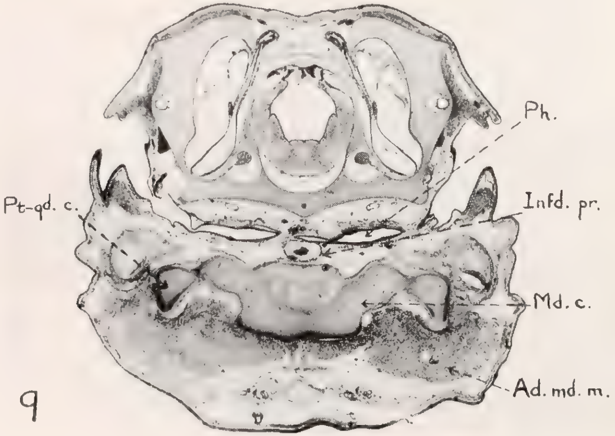

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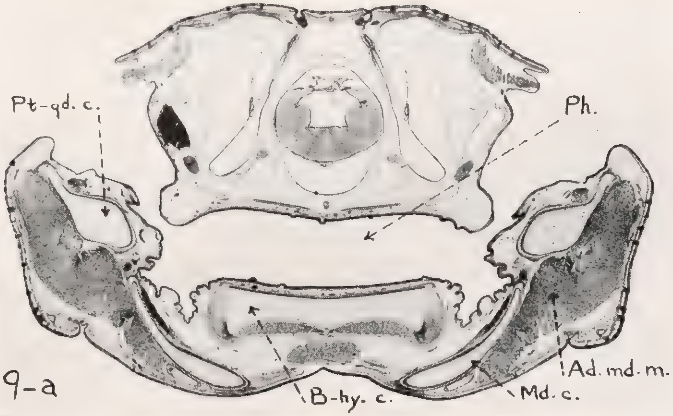

9-a

PLATE III

FIG. 8. Photomicrograph (× 4) of a section of the malformed specimen at the level of the spiracle (*Sp.*) showing the terminal bulbous enlargement of the infundibular process (*Infd. pr.*). The homologue of the pterygoquadrate cartilage (*Pt-qd. c.*) is associated with the conspicuous adductor mandibuli muscle (*Ad. md. m.*).

FIG. 8-*a*. A corresponding section of the normal control. At this level there is no part of the hypophysis.

FIG. 9. Photomicrograph of a section of the anomalous fetus at the level of the posterior edge of the spiracle showing the caudal end of the infundibular process in contact with the blind anterior end of the pharynx (*Ph.*). Mandibular cartilage (*Md. c.*).

FIG. 9-*a*. A corresponding section of a normal fetus. Pterygoquadrate cartilage (*Pt-qd. c.*). Mandibular cartilage (*Md. c.*). Basihyoid cartilage (*B-hy. c.*).

Furthermore, the interdependence in development of one part of the pituitary upon another may be quite different in mammals than in amphibia and fishes. The present case of pituitary agenesis in a lower vertebrate seems to bear out Smith's observations, for here, as in his tadpoles, in the total absence of the buccal ectoderm, the pars neuralis has not attained its normal shape nor has it undergone its typical differentiation into a saccus vasculosus.

Tadpoles in which the buccal hypophysis has been removed at an early stage of development display albinism in which the epidermal melanophores are diminished in number and pigment content besides remaining in a persistent state of contraction. These conditions in the tadpole are closely paralleled by this albino dogfish fetus in which the melanophores are less numerous, contracted, and noticeably withdrawn from the surface. Lundstrom and Bard, in a study of the effects of ablation of various parts of the brain of the dogfish (*Mustelis canis*), first discovered the hypophyseal control of the cutaneous pigmentation in the elasmobranch fishes. They found that removal of the neuro-intermediate lobe invariably resulted in pallor of the skin. The present anomalous specimen constitutes an interesting confirmation of their work. Evidently there has been a spontaneous suppression of the oral hypophysis equivalent to actual experimental ablation. Because of the intermingling of the elements of the pars neuralis and pars intermedia of the dogfish pituitary, it has so far been impossible to accomplish a complete operative separation of these two parts. The presence of the neuro-hypophysis in the present specimen, but the total absence of the oral pituitary (including the pars intermedia) indicates that the humoral agent affecting pigmentation is a derivative of the oral components of the gland. Presumably in that portion of the gland referred to as the neuro-intermediate lobe it is the buccal elements that are responsible for the chromatophore-expanding factor.

Observations in many cases of human anencephaly (Covell, 1927) make it apparent that aberrant formative processes involving defective closure in the dorsal midline may result in agenesis of the neural lobe of the pituitary. Cyclopia and astomia are occasionally found together in human monsters. It appears from the present observations that, in the dogfish, anomalous development in the ventral midline with imperfect separation of symmetrical parts and consequent cyclopia and astomia may result in agenesis of the oral hypophysis.

SUMMARY

1. An anomalous fetus of the spiny dogfish (*Squalus acanthias*) is described in which there are malformations of the head comprising cyclopia, astomia, and abnormalities of the hypophysis.

2. The abnormalities of the hyophysis involve:—

(*a*) The total absence of the oral components of the gland, and
(*b*) A neural lobe which is deformed and possesses no saccus vasculosus. The conclusion is drawn that the neural lobe has not undergone normal differentiation because it has been deprived of its usual association with the buccal hypophysis.

3. The specimen is albino, displaying a diminished number of chromatophores in a state of persistent contraction. This finding indicates that the melanophore-controlling principle in the dogfish is a derivative of the buccal components of the pituitary.

4. Only one other instance of total spontaneous suppression of the oral hypophysis is described in the literature, namely, in a pig fetus (Holt, 1921). In human fetuses anencephaly occurs not infrequently but is associated with suppression of the neuro-hypophysis instead of with the adenohypophysis.

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VARIATIONS OF COLOR PATTERN IN HYBRIDS OF THE GOLDFISH, CARASSIUS AURATUS

H. B. GOODRICH AND PRISCILLA L. ANDERSON [1]

(*From the Department of Biology, Wesleyan University*)

This paper gives an account not only of the differences between fish arising from the same genetic cross but also of the variations of color pattern taking place during the life of individual fish.

The cross between the common goldfish and the transparent shubunkin which are both varieties of the species *Carassius auratus* was first subjected to genetic analysis by Berndt (1925 and 1928) and Chen (1925 and 1928). The results indicated that the two parental types are genetically distinguishable by a single gene difference. The formulae as denoted by Chen are: common goldfish TT, the transparent shubunkin $T'T'$, and the hybrid TT'. This hybrid is known to the fanciers as the calico shubunkin. The common goldfish, which is quite brown or black during youth, changes to the familiar orange or red type by destruction of part or nearly all of its melanophores (Berndt, 1925; Goodrich and Hansen, 1931). This type also carries at least two layers of reflecting tissue, one beneath the scale layer and the other backing each individual scale. The transparent shubunkin has lost most of the chromatophores (both melanophores and xanthophores) and also most of the reflecting tissue. The heterozygous type, or calico fish, shows great variability in the distribution of both melanophores and xanthophores and there is no bilateral symmetry of pattern. A deep abdominal layer of reflecting tissue is present and a few scales are also backed with the tissue. For full details, papers by Chen (1928) or Goodrich and Hansen (1931) may be consulted.

Goodrich and Hansen (1931) made a detailed comparative study of the history of the melanophores of the three phenotypes covering the first eight weeks after hatching during which period the fish grew from 4.5 mm. to about 33 mm. in length. It was found that the history of the three types was similar for the first week (to 9 mm.) showing a uniform rate of multiplication of the chromatophores. After this the three types diverged. The normal goldfish showed a very rapid

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and uniform increase in number of chromatophores. In the transparent shubunkin the melanophores began to disintegrate until nearly all were destroyed. The hybrid, however, was found to be highly variable, showing great diversity between individuals. New cells appeared; others were destroyed. It gave the impression of a conflict between the cell proliferation and cell destruction.

Material and Methods

This paper continues the observations on the melanophore pattern of the heterozygous type beginning where the previous study was discontinued. The work was begun during the summer of 1937 with fish varying from 23 to 36 mm. in length (tip of mouth to base of caudal fin). The fish were chiefly obtained from the Grassyfork Fisheries of Martinsville, Indiana, to which institution we are greatly indebted. The hybrid fish were obtained directly from the hatchery which raises them regularly for the market. Records were made by photographing one side of the fish at intervals of approximately one month, but the periods were lengthened to longer intervals during the last six months. Ten of the fish are still under observation at this time, one year and six months after the start of the work. They vary from 47 to 58 mm. in length. All others that were started died. Anaesthetization, necessary for photography, proves to be fatal in some cases.

The individuals differ markedly from each other. For purposes of description two types, *A* and *B*, may be recognized, but it should be understood that there are intermediate gradations. Type *A* shows a relatively uniform distribution of melanophores on the dorsal half of the body and extending variably below the lateral line (Figs. 1 and 2). In Type *B*, the distribution of melanophores is much more uneven. They tend to be aggregated in clusters (Figs. 4 and 5). Xanthophores are present in both types and are unevenly distributed, but are not studied in this paper as it is very difficult to distinguish and identify the individual cells.

History of Color Patterns

Type A

It is possible with these fish to enumerate and reidentify from time to time all cells of large areas on the photographed side of the body. Except in the cases where wholesale destruction of melanophores occurs, it is found that few cells are lost and that individual cells have long life. An example may be taken (our fish number *MG*-3) on which 907 cells were enumerated and located on the side of the body (see

Table I for this and other references to cell counts). The first photograph was taken August 2, 1937 and the last February 17, 1939 making a total series of 18 photographs. During this time, 50 of the 907 cells disappeared and three new ones appeared. Figure 1 is the photograph taken August 2, 1937 and Fig. 2 that of January 4, 1939. The dotted lines outline arbitrarily delimited areas marked on the prints to facilitate the counting and identification of cells. The small circles indicate the former location of cells that have disappeared. Figure 2 is taken at a lower magnification than Fig. 1 and fish had grown from 26 mm.

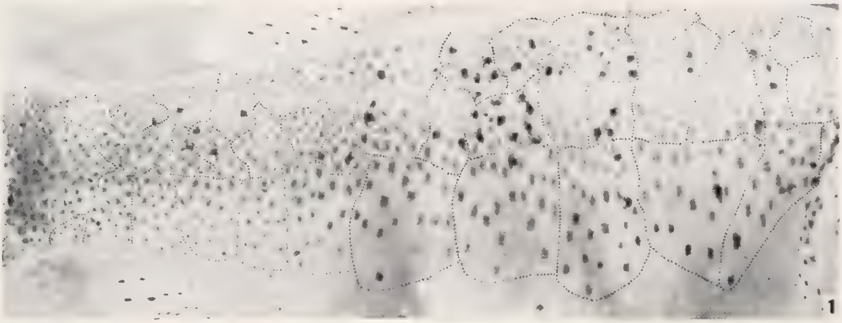
TABLE I

This table gives records of photographs of four of the fish studied. The dates are accurate only for *MG*-3 as it was not always possible to take all photographs on the same day.

	MG-3 Calico Type *A* 907 cells		*MG*-4 Calico Type *B* 97 cells, 2 cl.		*MG*-15 Transparent 21 cells		*MG*-16 Calico Type *A* 613 cells	
	D	*A*	*D*	*A*	*D*	*A*	*D*	*A*
July 17, 1937	0	0	0	0(4)				
Aug. 2	0	0(1)	9	1 cl	0	0	0	0
Aug. 26	1	0	6	2 1 cl	0	0	6	0
Sept. 20	5	0	2	0	0	0	1	0
Oct. 18	5	0	0	0	1	0	0	1 cl
Nov. 16	5	0	7	0	1	0	(6)	0
Dec. 15	4	0	4	1 cl	0	0		0
Jan. 26, 1938	2	0	3	4 cl	0	0		0
Feb. 23	4	0	7	2 cl	0	0		0
Mar. 28	1	0	1	3 cl	0	0		0
May 2	4	1	1	0	1	0		0
June 7	9	1	1	0	1	0	(7)	0
July 16	2	1	0	0	0	0		0
Aug. 5	2	0	1	0	0	0		0
Sept. 13	–	–	8	0	0	0		0
Oct. 12	4	0	1	0	0	0		0
Nov. 14	0	0	0	0	0	0		0
Jan. 4, 1939	0	0(2)	0	0(5)	0	0		0
Feb. 17	2	0	0	0	0	0		0
Totals	50	3	51	2 12 cl	4	0	All	1 cl

D—number of cells that disappeared since preceding photograph.
A—number of new cells appearing since preceding photograph.
cl—cell cluster or spot.
(1), (2), (4), (5) indicate pictures reproduced in Figs. 1, 2, 4, and 5.
(6)—time of beginning of wholesale destruction of melanophores.
(7)—time at which all melanophores were destroyed.

to 58 mm. in length (exclusive of caudal fin). Figure 3, however, shows the rectangular area of Fig. 2 raised to the same magnification as Fig. 1.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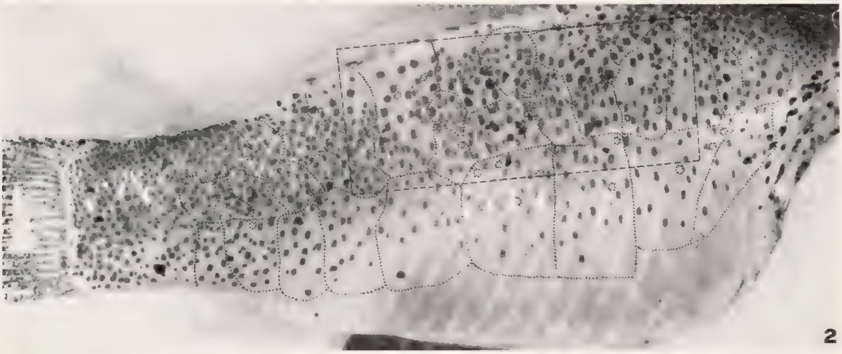
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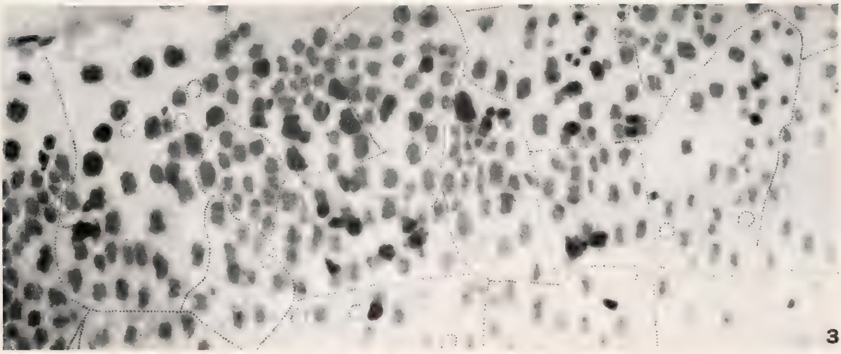
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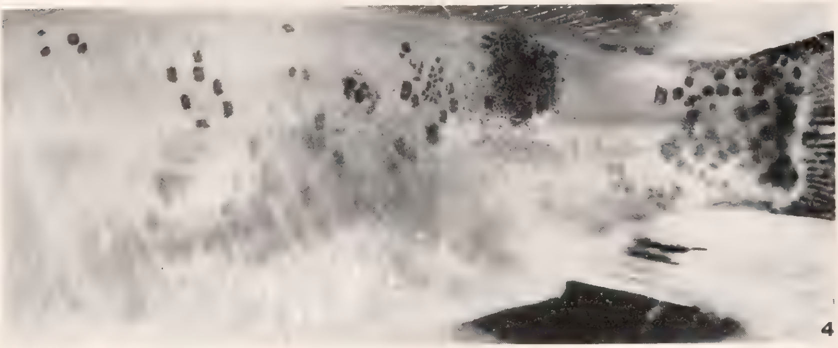
FIG. 1. Fish *MG*-3. Photograph taken August 2, 1937. × 5½. This is a "Type A" calico shubunkin; 907 cells are located in outlined areas.

FIG. 2. Fish *MG*-3. Photograph taken January 4, 1939. × 2½. Fifty-two cells have been lost and 3 new cells appeared since record of Fig. 1. Dotted circles indicate location of cells that have disappeared.

FIG. 3. Section outlined by dashes in Fig. 2 enlarged to same magnification as in Fig. 1, showing increase in size of area and of cells.

Type B

These fish show the irregular mottling which is prized by the fanciers. The dark spots are usually clusters of small melanophores too densely crowded to count. Of 97 selected on the first photograph of *MG*-4 on July 18, 1937, 46 remained on January 1, 1939 (Figs. 4 and 5). In the meanwhile, however, others have appeared and there has been a notable eruption of spots, or clusters of melanophores—12 altogether on the left side. These spots are first recognized as one or a few minute melanophores which rapidly increase in number. A spot for a time is often bounded by the posterior edge of a scale.



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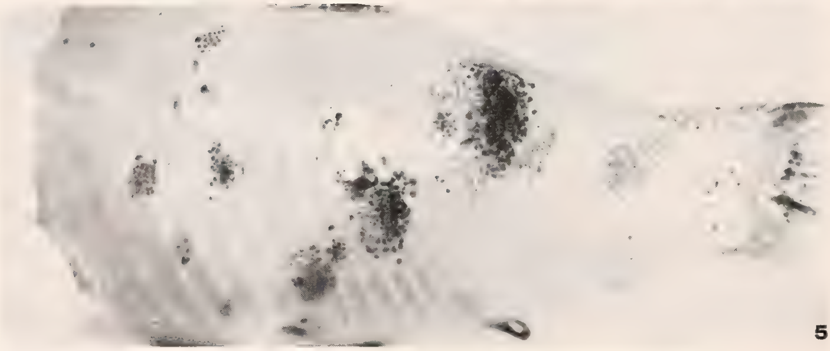

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FIG. 4. Fish *MG*-4. Photograph taken July 18, 1937. × 6½.

FIG. 5. *MG*-4. Photograph taken January 4, 1939. × 2½. Fifty-one cells disappeared and 12 new cell clusters appeared in area under observation.

Indeterminate Types

In many cases the clusters of small cells appear among, or superficial to, cells uniformly distributed and in this way combine characteristics of Types *A* and *B*. An example is *MG*-5, where it was possible to identify cells only in a small area. Ten disappeared out of 164 in this area, but six new clusters of cells have arisen similar to those discussed under Type *B*.

Extensive Cell Destruction

Occasionally a sweeping destruction of melanophores occurs within a few weeks. This is similar to the process in the ordinary goldfish, which is gold because melanophores but not xanthophores have been destroyed. This change most frequently takes place in ordinary goldfish at about three months of age but may occur much later (Cf. Berndt, 1925; Chen, 1925; and Goodrich and Hansen, 1931). It occurred in two of the calico shubunkins which we had under observation in this series. The history of one of these, *MG*-16, is given in Table I and in this the breakdown occurred at about eight months of age.

Increase in Size of Cells

As mentioned above, Fig. 3 shows the rectangular area marked on Fig. 2 enlarged to the same magnification as Fig. 1. The comparison of Figs. 1 and 3 then shows the actual increase in size of the area outlined. It also shows that the individual cells, which for the most part show approximately the same degree of melanin dispersion in both pictures, have definitely increased in size. It, therefore, appears that, in so far as the melanophores are concerned, the increase of body size has involved an enlargement of cells rather than a multiplication of cells.

Discussion

These observations not only show that there is much variation among individuals of these hybrids but also that each individual is variable in respect to color patterns displayed during its life cycle. The heterozygous type, as noted for earlier developmental stage by Goodrich and Hansen (1931), continues in later stages to be in a condition of unstable equilibrium between opposing tendencies—those of cell multiplication and cell destruction.

Fukui (1927 and 1930) has shown that the destruction of melanophores in the ordinary goldfish tends to take place in definitely bounded areas, giving rise to some degree of uniformity of pattern in black- and goldfish. These areas, he believes, correspond to regions of looser subcutaneous tissue bounded by more dense tissue. In effect, these may be perhaps regarded as sinuses filled with tissue fluids or lymph.

His experiments with injection of adrenalin showed a restoration of pigment which tended to be circumscribed in such areas. These results suggest that endocrine factors operating on such a region bring about under certain conditions the destruction of chromatophores and under other conditions the production of pigment. Fukui suggests that pigment destruction is due to a higher metabolic rate in these areas, but this might be stimulated by the chemical environment.

In contrast to the above, the origin of new spots or cell clusters is entirely irregular, having no relation to the areas described by Fukui. It, therefore, seems unlikely that their location can be due to endocrinal conditions.

It then seems probable that the goldfish presents a new example of the dual gene control such as has been suggested in the plumage of birds. In the case here described the direct gene action may control cell multiplication, resulting in the formation of cell clusters or spots, while remote gene control of "endocrinal regulation" may cause the destruction of cells (see Danforth, 1932, p. 33).

A discussion of the developmental origin of cell clusters will be presented in the companion paper, Goodrich and Trinkaus (p. 188).

SUMMARY

1. The F_1 heterozygous types from the cross of the common goldfish with the transparent shubunkin (both of the species *Carassius auratus*) show not only a great range of variability between individuals, but frequently the pattern of a single individual changes markedly during the life cycle. This is due to destruction and emergence of chromatophores producing a varying pattern. It is suggested that the multiplication of cells is an example of "direct gene control" and the destruction is due to "endocrinal regulation" or remote gene control.

2. Many individual melanophores are long-lived, having been identified at the beginning and end of the 19-month period of observation.

3. Such long-lived melanophores gradually increase in size during the growth of the fish.

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THE DIFFERENTIAL EFFECT OF RADIATIONS ON MENDELIAN PHENOTYPES OF THE GOLDFISH, CARASSIUS AURATUS[1]

H. B. GOODRICH AND J. P. TRINKAUS

(From the Department of Biology, Wesleyan University)

The types of goldfish used in the following experiments are those described in the companion paper by Goodrich and Anderson (1939). These are the common goldfish, the transparent shubunkin, and the hybrid between these two known as the calico shubunkin. Genetic analysis has shown that this is a monohybrid cross and the formulae assigned have been: ordinary goldfish TT, the transparent shubunkin $T'T'$, and the calico fish TT'.

The original purpose of the ultraviolet treatment was to destroy certain parts of the color pattern in the calico fish and to study its regeneration. It was, however, soon discovered that lighter treatment than that needed to destroy the chromatophores apparently induced the formation of new pigmented areas. Consequently a more careful program of experimentation was outlined to verify these preliminary findings.

METHODS

The source of illumination has been a small laboratory mercury lamp obtained from the Hanovia Company (their model E). The quartz tube is 16 mm. in diameter, has a length of arc of 50 mm., and operates on 110-volt circuit. For purposes of destruction of melanophores, treatments frequently of 30 minutes or more were administered, but for stimulation of pigment formation most treatments were of 10 minutes duration at distances varying from 2 cm. to 6 cm. from the lamp. Only a small area was irradiated on each fish. Other parts of the body within the zone of illumination were protected. The areas treated varied from about 0.2 to 0.9 sq. cm. in size. These were delimited by pieces of wet filter paper over which were placed pieces of tin foil, which in turn were held in place by more filter paper. Wet cotton was put over the head and the operculum and over the rear of

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[1] This paper is published as part of a research program at Wesleyan University supported by the Denison Foundation for Biological Research. The authors wish to acknowledge their indebtedness to Miss Priscilla Anderson who performed the preliminary experiments.

the body and caudal fin. This kept the fish moist and helped to hold it in place. The fishes were anesthetized in a 1 per cent urethane solution and were placed on a paraffin block modeled to hold the fishes nearly upright. During irradiation the spot treated was kept wet with distilled water to prevent drying of the tissue. Photographs of both sides of the fish were taken before treatment. The irradiated areas were outlined on the photographic prints and later the location of new spots was marked on these prints, or additional photographs taken if thought desirable. The fish were inspected at weekly intervals for the first three months after the treatment and those fishes that survived were observed at longer intervals for the succeeding six months.

Experiments

After the preliminary experiments, it was first planned to treat approximately equal numbers of the three Mendelian types. Accordingly, ten of each were irradiated. Later, the numbers treated were increased, especially of the hybrid type which was the only form which gave a positive reaction. The final lot of fish irradiated included 24 of the ordinary goldfish TT, 17 of the transparent shubunkin $T'T'$, and 52 of the hybrids TT', giving a total of 93 fish treated.[2] Areas with few or no melanophores were selected for irradiation. The essential result from the comparative study was that the hybrids alone showed a positive reaction by development of new melanophores, while in the two parental types no melanophores were formed. Most fish in all these groups exhibited inflammation and sometimes necrosis of tissues. In the goldfish TT the xanthophores and guanin crystals (of the reflecting tissue) were frequently destroyed. Spots or cell clusters appeared only in the hybrids. These were first observed as small faintly grayish chromatophores, having long delicate processes. The number of cells increased and in about eight weeks these cells became typical mature goldfish melanophores. (Figure 3 shows the inflammation following irradiation, and Figs. 4 and 5 the development of a cell cluster in the same spot.) Figure 1 is a photograph of a hybrid TT' taken on March 29 just before radiation and the area irradiated is outlined. Figure 2 is of the same fish on May 27. Three new spots, one small and two large, have appeared in the radiated area and one outside (in dotted circle). All but one of the new spots were located in the dermis superficial to the scales. This one exceptional spot was beneath the scales.

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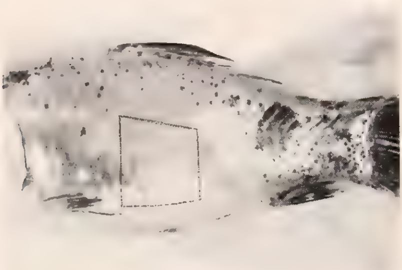
[2] Eight fish of doubtful classification are excluded from these totals. Inspection of pattern indicated that they probably were one normal goldfish and seven transparent shubunkins. All gave negative reactions. Even if the presumed transparent types were transferred to the list of 52 calico shubunkins the essential results as indicated by the graphs, Figs. 6 and 7, would not be altered.

The companion paper (Goodrich and Anderson, 1939) has shown that the hybrid or calico fish is characterized by an irregular mottling and, moreover, that this pattern is subject to change during the life of the individual. It therefore seemed possible that the appearance of new spots after radiation might be nothing more than the normal sequence of events. On this account, many more of the hybrids were treated and the results subjected to analysis. This has shown that the irradiated areas produced a significantly greater number of spots or cell clusters than appeared on non-radiated areas. It was also found that the new spots appeared chiefly from three to six weeks after treatment with the maximum number arising during the fifth week (see charts, Figs. 6 and 7). In 24 cases two or more cell clusters appeared within the radiated area, in 17 cases only one new spot and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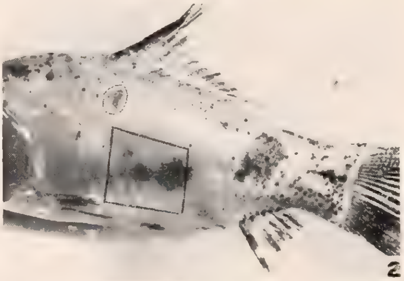
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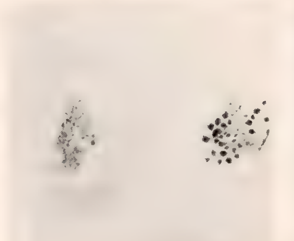
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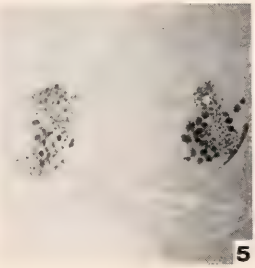
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FIG. 1. Photograph of a hybrid TT' taken on March 29 just before radiation. The area later irradiated is outlined with dotted line. × 1¼.

FIG. 2. Photograph of same fish as in Fig. 1 taken on May 27. Three new spots (one small and two large) have appeared in the radiated area and one outside (in dotted circle). × 1¼.

FIGS. 3, 4, 5. Successive photographs of the same area on a hybrid fish. × 6. Irradiation Nov. 13, 1937. Fig. 3, appearance Nov. 27; congestion of capillaries in center (an older spot at right). Fig. 4, Jan. 2, 1938. Fig. 5, Jan. 25, 1938.

none were recorded in 11 cases. These results have been compared with the total number of cell clusters appearing on both sides of the body (exclusive of head and fins). The results appear significant even

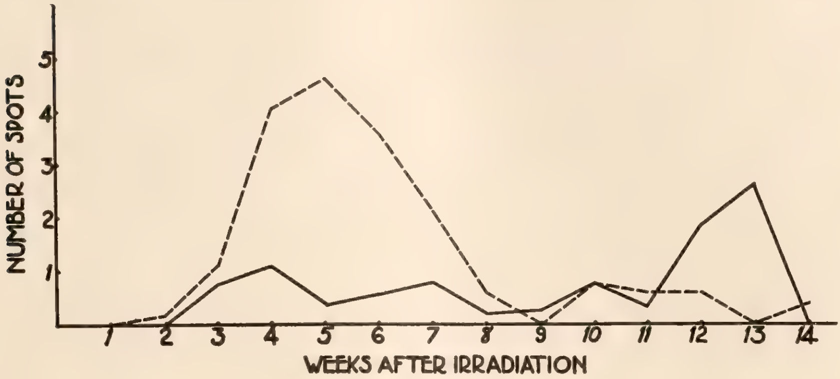

FIG. 6. Graph of numbers of spots X10 appearing in successive weeks after irradiation. Dotted line, irradiated area. Solid line, other parts of body (head and fins not included).

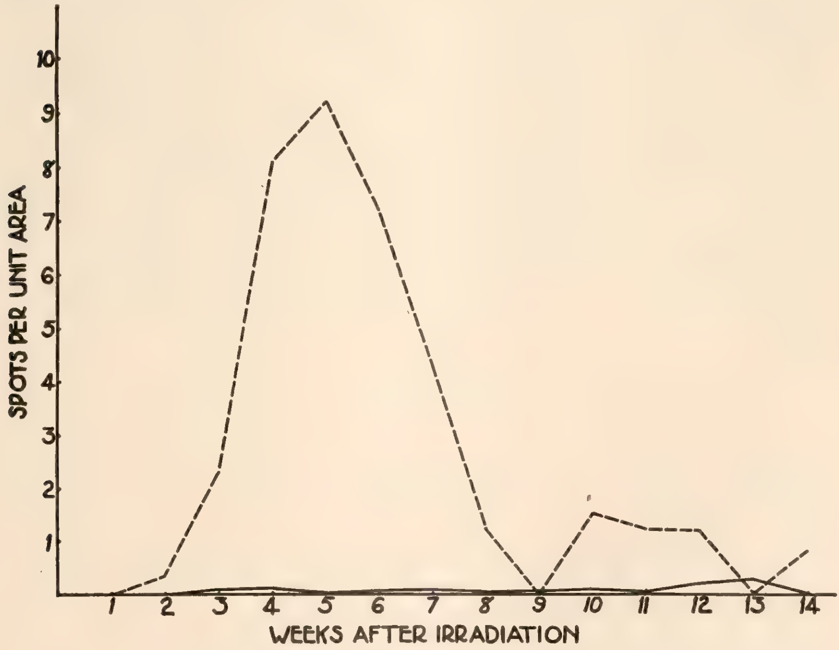

FIG. 7. Graph showing same data as Fig. 6 corrected for relative size of areas. Dotted line, numbers in irradiated area X20. Solid line, numbers on other parts of body X-1.

when no correction is made for the difference in areas compared. When, however, such a comparison is made it is found that the total non-radiated surface examined was approximately twenty times that of the average irradiated area. A graph, incorporating this correction, is shown (Fig. 7) and indicates a notable excess of development of spots in the irradiated areas. During the observational periods, from treatment until 14 weeks thereafter, there appeared a total number of 62 new spots or cell clusters within the irradiated areas and 32 outside of these areas. If we multiply by the factor 20 ($20 \times 62 = 1240$), it appears that had spots appeared at a similar rate in the non-radiated region there would have been 1240 spots, whereas there were only 32. This proportion of nearly 38 : 1 is then an index of the increased reaction of the radiated region. It is not impossible that this is an underestimate. The areas chosen for treatment were frequently below the lateral line, because this region was more clear of melanophores, and it is possible that the ventral region is one having less inherent capacity for production of melanophores.

The new cells recorded in the above experiments were in all respects similar to normal melanophores present elsewhere on the fish. Two sets of subsidiary experiments were carried out which, incidentally, gave further confirmation that these cells were normal melanophores. (1) It was found that the melanophores of the hybrid responded very irregularly to an illuminated white environment. In some cells the pigment became concentrated and in others it remained dispersed. New cells arising in the irradiated areas showed this same variability of reaction. (2) Ten scales bearing new cell clusters were transplanted to other parts of the fish as had previously been done by Goodrich and Nichols (1933) with non-radiated fish. The results were similar. The cells lived and in four cases increased, spreading over adjoining scales.

Discussion

The observations presented in this and the preceding paper (Goodrich and Anderson, 1939) show that the hybrid or calico shubunkin retains the potentiality to produce irregularly situated spots during a considerable part of the life cycle. The radiation appears to stimulate a precocious development of the spots in the areas treated. The question then arises as to what developmental or other conditions control the appearance of these spots or cell clusters. Goodrich (1927), working on the Japanese fish *Oryzias latipes*, suggested that the variegated pattern could be explained by the ameboid migration of predetermined melanoblasts of two types—that producing the maximum amount of melanin and the other such a small amount that they remained virtually colorless. Recent investigations such as those of DuShane (1935) and Twitty (1936) on amphibia have tended to confirm the hypothesis of an early determination of wandering chromatoblasts. The paper by Willier and Rawles (1938) on the chick opens the possibility of cell determination and migration in forms where hormones have been shown to be largely operative in other phases of pigment control. The observations of Apgar (1935) on *Triturus* have suggested the concept of a widespread distribution of colorless chromatoblasts. It, therefore, seems not improbable that we may consider the calico shubunkin (especially Type *B* of the companion paper) to be invisibly spotted during development with colorless chromatoblasts—singly or in nests—and that these multiply and differentiate independently at irregular intervals to form the spots or clusters of melanophores. In some respects this hypothesis resembles the old theory of embryonic cell rests advanced to explain the cause of cancer.

In certain individual fish a wave of destruction takes place, possibly due to some hormone action, which destroys all melanophores and possibly all melanoblasts in the affected areas. We have never observed the appearance of new spots in a region which has suffered such wholesale destruction.

Attention should be called to the production in goldfish of pigment cells by X-rays (Smith, 1932). The cells appeared within a few days after treatment and disappeared a few weeks later. They did not seem to be homologous to the pattern-producing cells and resembled cells that had previously been observed arising after various mechanical injuries to the tissues (Smith, 1931). In our own experiments we have noted three cases of the formation of such cells. They were seen on the normal goldfish *TT* after unusually severe radiation from the mercury arc lamp and the appearance and history of these cells were similar to those noted by Smith.

The contrasting reactions of the three genotypes indicate that the hybrid or calico fish retains in adult condition a far greater potency to produce melanophores than either parental form. Goodrich and Hansen (1931) have pointed out that all three types form melanophores in early development. The ordinary goldfish loses these by wholesale destruction usually at about three months of age, while in the transparent shubunkin relatively few ever appear. Neither of these two parental forms produced typical melanophores when irradiated and it may be suggested that melanoblasts have also been destroyed or are largely absent. In contrast, the heterozygous type retains the melanoblasts.

No attempt is made in this paper to determine what wave-lengths have produced the observed effect. The mercury vapor arc produces a wide range of wave-lengths. The extensive literature on effects of ultraviolet light shows that both stimulating and destructive effects have been observed. Sperti, Loofbourow, and Dwyer (1937), working on yeast cells, have suggested that cells when injured by ultraviolet liberate some growth-promoting substance, thus indicating a possible interrelation of injurious and stimulating effects. The treatments used in our experiments have been relatively more severe than those which have produced primarily stimulating effects on isolated cells. Ultraviolet light penetrates but a few millimeters through animal tissues. Sato (1933) has shown that the ultraviolet light bands characteristic of the mercury arc will pass through fish scales. The effect produced in our experiments may well be due chiefly to the regenerative processes following the inflammation and destruction of tissue.

SUMMARY

1. Radiation from a mercury vapor lamp produced differing reactions in three Mendelian phenotypes. Two parental forms, the ordinary goldfish and the transparent shubunkin, do not develop melanophores as a result of the treatment. The F_1 hybrid, or calico shubunkin, does respond by an acceleration in the production of new spots or clusters of melanophores.

2. It is suggested that the hybrid during development becomes supplied with colorless chromatoblasts throughout the dermis which are stimulated to precocious multiplication and differentiation as a result of the radiation.

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THE REACTIONS OF THE PLANKTONIC COPEPOD, CENTROPAGES TYPICUS, TO LIGHT AND GRAVITY[1]

W. H. JOHNSON AND J. E. G. RAYMONT

(*From the Department of Physiology, McGill University, the Biological Laboratories, Harvard University, and the Woods Hole Oceanographic Institution*)

Introduction

Field investigations on the vertical distribution of the plankton carried out by many different workers in recent years have established the occurrence of a diurnal vertical migration for most species of the zoöplankton. Since most investigators agree in considering light as an important controlling factor, it seemed desirable, following the work of Esterly, Spooner, Clarke and others, to attempt to study the light responses of a single planktonic species under controlled laboratory conditions.

Centropages typicus is a neritic copepod, extremely abundant off Woods Hole at certain times of the year. Clarke (1933) states that the adults show a diurnal vertical migration correlated with changes in the submarine illumination. A few preliminary observations in the laboratory showed us that the adult females are very definitely affected by light. It was therefore decided to conduct experiments on the phototropic and geotropic responses of these animals. Our choice was fortunate in that it was possible to obtain the copepods quickly and easily off Woods Hole, and to keep them at a conveniently low temperature in the laboratory to ensure their healthy existence for at least a few days.

Phototropism: Experiments with Tubes Horizontal

In order to separate the phototropic from possible geotropic responses, it seemed advisable to test first the reactions of the copepods to light in a horizontal direction.

Methods

The experimental animals were obtained in Vineyard Sound by towing a scrim plankton net horizontally near the surface for about fifteen minutes. The animals, collected in the glass jar attached to

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[1] Contribution No. 207 from the Woods Hole Oceanographic Institution.

the net, were poured into 3 liters of sea water and transported immediately to the laboratory. The adult female *Centropages typicus* were selected in diffuse daylight using a wide-mouthed pipette and a binocular microscope. Usually 20 healthy appearing copepods were placed in each of two glass tubes (13 × 2½″), each of which was sealed at one end with a glass plate. The open ends of the tubes were then sealed with similar glass plates, and the tubes arranged in constant temperature tanks maintained at 12° C. in the darkroom.

The two experimental tubes could be separated from each other by a distance of 21 feet. It was thus possible to obtain a wide range of light intensities for any one source, by varying the distance between the light source and the tubes. The intensities of the various inside frosted bulbs employed were as follows:[2]

Wattage	*Approximate Intensity at 1 foot*
15	13.5 foot-candles
25	25.0 "
40	43.0 "
60	75.0 "
100	150.0 "

It should be borne in mind that all the light intensities mentioned in the text are only approximate figures.

The lowest intensities used were obtained by means of neutral filters in the form of opal discs and white paper, the percentage absorptions of which were obtained by means of a photoelectric cell. Since several filters were used together at the very lowest intensities, corrections were made for diffusion and back-scattering.

Each experimental tube was marked off into quarter-lengths, and the distribution of the animals at any time, under any one condition of light, was expressed as the numbers in each section. At the very low light intensities, counting of the copepods was facilitated by lighting the tubes from behind for a moment with a weak red lamp. Preliminary tests made with this lamp showed that it had no effect on the distribution of the animals.[3]

In all the experiments, unless otherwise noted, the distribution of the animals was observed at the end of each time interval shown in the tables. After each observation, the tubes were changed, end for end, by turning them slowly in a horizontal plane. This procedure forced the animals to orientate afresh, and to redistribute themselves according to the tropistic responses actually in operation during that time interval. Enough time was allowed for the animals to establish their new distribution before a second record was taken, so that their final position was unaffected by the configuration of the previous time interval.

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[2] On the advice of Mr. Eddie Kline, electrical engineer of the Canadian Laco Lamp Co., these can be considered as accurate only within 20 per cent, due to voltage fluctuation.

[3] Dr. Horton of the Department of Physics, McGill University, kindly made a spectroscopic photograph of the light emitted and found that the transmission begins at 6402 Å, and continues beyond 8600 Å.

TABLE I

Experiment commenced at 4:00 P.M., August 19.
Tubes *A* and *B* set at distance of 5 ft. and 1 ft. respectively, from source.
At 12:00 noon, August 20, tube *A* moved to 10 ft.
At 4:00 P.M., August 20, tube *A* moved to 20 ft.
Tube *B* was kept at 1 ft. throughout.
Source: 60-watt lamp.

Time	Distance	Intensity	(Positive) * I	II	III	(Negative) IV
Aug. 19 4:10 P.M.	5 ft.	3.0 f.c.	20	–	–	–
	1 ft.	75.0 f.c.	20	–	–	–
4:40 P.M.	5 ft.		20	–	–	–
	1 ft.		20	–	–	–
6:45 P.M.	5 ft.		16 and 4	–	–	–
	1 ft.		20	–	–	–
9:15 P.M.	5 ft.		10 and 6	–	2	3
	1 ft.		10 and 3	3	–	1
9:30 P.M.	5 ft.		8 and 6	–	2	2
	1 ft.		3 and 7	3	3	2
Aug. 20 11:30 P.M.	5 ft.		10 and 3	1	4	3
	1 ft.		4 and 8	3	1	1
2:30 P.M.	10 ft.	0.75 f.c.	8 and 3	–	3	3
	1 ft.	75.0 f.c.	5 and 9	3	1	1
4:00 P.M.	10 ft.		8 and 4	4	4	–
	1 ft.		3 and 7	4	1	1
4:45 P.M.	20 ft.	0.19 f.c.	9 and 6	–	3	–
	1 ft.	75.0 f.c.	5 and 10	3	2	1
8:00 P.M.	20 ft.		6 and 9	–	2	2
	1 ft.		3 and 9	4	1	2

* Two numbers are sometimes given under Section I (e.g. 16 and 4). This distinguishes those copepods right at the positive end (16), from those still in this section but apparently less strongly attracted.

Observations

A series of tests (Table I) was first carried out in order to determine:

(1) The normal responses of the copepods to various light intensities within limits found in nature.
(2) The effect of continued exposure to constant light intensities over the range studied.

The results obtained (Table I) showed that the copepods were positive to all illuminations, and remained largely so after exposure.

A number of experiments was then carried out to determine the range of light intensities to which the copepods were sensitive, and to investigate the possibility of the existence of critical light intensities at which the phototropic sign might become reversed.

The copepods were found to be positive to low light intensities, the lowest to which they were attracted being *ca.* 0.005 f.c. (Table II).

TABLE II

Responses to low light intensities. Distance of experimental tube from source: 20 ft. throughout.*

Intensity	Time	(Positive) I	II	III	(Negative) IV
0.06 f.c.	Aug. 20 9:30 P.M.	5 and 4	–	2	3
" "	Aug. 21 8:30 A.M.	4 and 6	1	2	1
0.015 "	10:45 A.M.	0 and 12	–	3	1
" "	12:00 Noon	2 and 12	1	2	3
" "	3:30 P.M.	4 and 5	1	4	–
0.008 "	Aug. 24 12:40 P.M.	14	1	3	2(New animals)
" "	2:00 P.M.	1 and 10	2	2	3
" "	3:00 P.M.	3 and 9	1	3	3
0.006 "	3:30 P.M.	11	2	1	3
" "	4:50 P.M.	6	5	–	7
" "	7:00 P.M.	11	2	2	1
" "	8:00 P.M.	10	–	2	7
" "	10:50 P.M.	13	–	4	1
" "	Aug. 25 8:30 A.M.	15	1	–	–
" "	9:30 A.M.	14	–	2	1
0.005 "	Aug. 26 12:30 P.M.	9	5	4	1
" "	1:55 P.M.	10	4	3	–
" "	2:25 P.M.	3	7	6	3
0.003 "	Aug. 28 2:30 P.M.	3	4	7	6(New animals)
" "	4:45 P.M.	9	4	4	3
" "	Aug. 29 9:40 A.M.	11	6	2	–
" "	11:50 A.M.	4	6	3	6
" "	6:45 P.M.	8	3	3	3

* Each time the light intensity was changed, it was done immediately following the preceding observation.

On continued exposure to the much higher light intensities of 150 and 600 f.c. (Table III), the majority of animals on the whole exhibited a positive phototropism, although at times there were more animals in the darker half of the tube and some of the animals apparently became negative on prolonged exposure.

It seemed desirable to determine whether the copepods would be repelled by the still higher light intensity (11,380 f.c.) approximating to that of bright sunlight. As a check on the results, other copepods which had been collected at the same time were subjected to a much lower intensity of 4 f.c. The results (Table IV) show that, at least after a short exposure to this very high intensity, half of the animals became negatively phototropic, while the others remained positive.

Table III

Responses to high light intensities
Source: 100-watt lamp. Intensity at ½ ft. : 600 f.c.
Intensity at 1 ft. : 150 f.c.

Time	Distance	(Positive) I	II	III	(Negative) IV
Aug. 30 5:00 P.M.	½ ft.	15	–	–	3
	1 ft.	14	–	2	3
5:30 P.M.	½ ft.	13	1	1	3
	1 ft.	13	–	1	6
6:45 P.M.	½ ft.	14	1	2	1
	1 ft.	10	1	–	8
9:00 P.M.	½ ft.	14	–	1	2
	1 ft.	14	1	2	3
10:15 P.M.	½ ft.	14	2	2	–
	1 ft.	10	–	1	8
Aug. 31 9:10 A.M.	½ ft.	5	1	2	10
	1 ft.	12	3	3	2
10:20 A.M.	½ ft.	8	1	3	7
	1 ft.	10	–	2	8
11:15 A.M.	½ ft.	11	2	1	4
	1 ft.	9	–	2	8
12:15 P.M.	½ ft.	9	1	1	8
	1 ft.	10	2	3	6
1:20 P.M.	½ ft.	12	2	–	4
	1 ft.	12	2	2	4
4:00 P.M.	½ ft.	12	2	–	4
	1 ft.	8	2	2	8
5:00 P.M.	½ ft.	12	1	3	3
	1 ft.	11	2	–	7

It was rarely that all the animals displayed an invariable reaction (either positive or negative) to any one condition of light. It was possible then that some of the animals were negatively phototropic even though the majority were positive; or again, perhaps some were indifferent. To gain evidence on these points, observations were made on individuals, one being sealed within a tube. At first, observations were made for the most part once every hour, using three widely separated intensities: 3.0, 150, and 600 f.c.

At the lowest intensity (3.0 f.c.), an individual remained photopositive for four hours, but appeared to become indifferent after exposure overnight. A second individual was indifferent from the first, and remained so for 15 hours. This behaviour was not modified if the individual was left in darkness, and then exposed to the light.

TABLE IV

Source: 1000-watt lamp. Tube at $4\frac{1}{2}$ inches from source.
Control tube at 20 feet.
Ice added to aquarium to offset intense heat from source.

Time	Intensity	(Positive) I	II	III	(Negative) IV
11:40 A.M.	11,380 f.c.	10	–	2	6
	4 f.c.	8	4	1	6
11:55 A.M.	11,380 f.c.	12	–	–	6
	4 f.c.	11	2	–	5
12:05 P.M.	11,380 f.c.	8	1	–	9
12:20 P.M.	11,380 f.c.	10	–	4	6
	4 f.c.	13	–	2	3
1:20 P.M.	11,380 f.c.	8	1	2	10
	4 f.c.	11	2	2	3
1:35 P.M.	11,380 f.c.	9	1	3	8
1:45 P.M.	11,380 f.c.	8	1	3	8
	4 f.c.	15	2	2	–
2:00 P.M.	11,380 f.c.	9	–	–	9
2:10 P.M.	11,380 f.c.	10	1	1	7
	4 f.c.	14	2	1	1
2:25 P.M.	11,380 f.c.	11	1	–	7
2:40 P.M.	11,380 f.c.	8	1	–	11
	4 f.c.	14	2	1	1

The responses of two individuals at an intensity of 150 f.c., and of two others at 600 f.c. were such that one individual at each intensity remained positive for 24 hours, while the other individuals were positive for the first 5 hours but apparently became indifferent after exposure overnight.

More extensive experiments on individuals were carried out, making observations every ten minutes, so long as it was possible to do so, over a long period of time, and at a wide range of intensities (600, 150, 75, 67, 33, 13.5, 2.4, 0.87, 0.03, 0.006, and 0.002 f.c.). Of four individuals (*A*, *B*, *X*, and *Y*), specimens *B* and *Y* were strongly and constantly photopositive to all the above intensities; indeed, specimen *B* was never recorded outside Section I. Individual *A* was in the main attracted although less so at intensities above 75 f.c. Individual *X*, although less consistent, was generally attracted by the light, but occasionally at both high and low intensities it was found at the negative end of the tube, even from the beginning of the experiment.

Having studied the effects of continued exposure to different intensities, it was decided to determine the effect of changing light intensity—a condition which is more like that which occurs in nature. The changes in intensity were obtained by varying the position of the source relative to the two experimental tubes. Thus the quality of the light remained unchanged, and two experiments could be carried on at once.

Successive experiments were carried out by moving the source first 1 foot, then 2, 5, 10, and finally 20 feet every ten minutes (owing to difficulties in counting, 15-minute intervals were sometimes unavoidable). The intensities ranged from 11,380 to 4 f.c. Before the experiments were commenced, the tube at the maximum intensity was left exposed to light until a considerable percentage of the animals exhibited repulsion.

Regarding the one-foot changes: On increasing the intensity from 4 to 11,380 f.c., the animals remained continually attracted showing always at least 80 per cent in the positive half of the tube. However, after continued exposure for one hour at the highest intensity, only 40 per cent were still attracted. In the opposite tube, 55 per cent of the copepods were repelled at the beginning when the intensity was 11,380 f.c., and it was necessary to decrease the intensity to 64 f.c. before 80 per cent of the animals were attracted.

Considering the results of the 2 ft. changes, it was found that essentially similar conclusions could be reached. In the increasing intensity experiment, actually 100 per cent of the animals exhibited constant positive phototropism. Decreasing the intensity resulted in progressive attraction down to 16 f.c., when about 80 per cent of the animals were in Sections I and II. Further decreases caused little change.

The 5, 10 and 20-foot changes may be considered together. Regarding the increasing intensities, it is striking that none of the changes had any effect on altering the original distribution of the animals. The numbers of animals in each half of each tube remained almost perfectly constant with the ten-minute intervals allowed, and it was only after prolonged exposure (45 to 60 minutes) at 11,380 f.c. that repulsion was brought about. Of the decreasing intensity experiments, in the 5-ft. changes progressive attraction resulted in 80 per cent of the animals being positive at an intensity of 7 f.c. Progressive attraction also resulted in the other experiments, with 70 per cent of the animals being attracted in the 10-ft. changes at the minimum intensity of 4 f.c. (After one hour at 4 f.c., 80 per cent were positive.)

All these experiments on different magnitudes of decrease, each occurring with 10-minute intervals, would seem to indicate that the greater the magnitude of change, the lower the intensity at which a large number of the copepods became positively phototropic. This statement may be misinterpreted unless it be remembered that undoubtedly 80 per cent, or more, of the copepods would have migrated to the positive half of the tube at much higher intensities had more time been allowed before the next change was made. (There would thus appear to be a "time-lag" effect.)

The above experiments show the effects of different magnitudes of increase and decrease with a constant time interval of 10 minutes. The percentage relationship between any one intensity and that which immediately preceded it is not by any means constant during any one succession of changes. Thus experiments were next conducted similar to the foregoing, except that there was a constant percentage increase or decrease throughout each series of changes. The actual rates of change used were such as may occur in nature. (The values chosen were the maximal changes observed by Clarke (1933) at one station in the Gulf of Maine.)

Increases and decreases of 10 per cent per hour were first tried, through a range of high intensities (11,380 to 2,840 f.c.), and then through a low intensity range (9.5 to 6.2 f.c.). Considering first the decreasing intensities, through the high range there was progressive attraction, while through the low range there was practically no alteration in the distribution. As regards the increasing intensities experiment, there was little observable change, but, if anything, a rather larger percentage of animals was attracted with time. The same result was obtained with the low intensity range. Decreases and increases of 20 per cent per hour, through both high and low intensity ranges, gave similar results.

Phototropism and Geotropism: Experiments with Tubes Vertical

Parker, Dice, Esterly, Clarke and others have demonstrated that geotropism is frequently an important factor in the vertical migration of plankton. It seemed desirable, therefore, to carry out experiments using vertical tubes to ascertain whether the light responses would be different, and to test for the occurrence of a true geotropic reaction.

Methods

The aquaria were replaced by two large bell-jars held upright by specially constructed wooden stands. The same experimental tubes were used, but they stood vertically in all the following experiments. A lamp was suspended over each tube, and, by means of a pulley system, the distance between the lamp and the tube could be quickly altered. The maximum distance thus obtainable was 4½ feet. Whenever it was desired to illuminate the animals from below, the tubes were simply placed upright on an iron tripod, and the lamp placed underneath.

Observations

It was decided to find the effects of various rates of change of light intensity, and to compare the results with those obtained in the horizontal experiments. Unfortunately the 1,000-watt lamp burned out and as it was impossible to replace it in the short time remaining, it was necessary to confine the indoor experiments to the lower light intensities (0.67 to 240 f.c.). A wide variety of rates of change was used: 25 per cent per hour, and 25, 50, 100, 300, and 700 per cent per half-hour.

Considering the experiments on increasing light intensities the following conclusions were reached. Within the range of intensities used, it seemed that, in general, increasing the light at a variety of rates does not bring about repulsion. One experiment, however, using 25 per cent increases per half-hour, through a range from 7.4 to 19.1 f.c. did cause repulsion:—70 per cent of the copepods were attracted initially, but as the intensity increased, fewer remained positive until only 16 per cent were attracted at 19.1 f.c. A large number of other experiments, however, at intensities near 7.4 to 19.1 f.c. (also at higher and lower ranges, and at rates from 10 per cent to several hundred per cent) was carried out, and in no other case was this repulsion observed. In the great majority of cases the distribution remained almost constant. It may be then, that this single case of repulsion does not demonstrate the normal behaviour of these animals, at least under laboratory conditions.

In the experiments on decreasing light intensities, with the exception of a single experiment, decrease in intensity at all rates, and through all the ranges of intensity employed, resulted in more and more of the animals swimming to the top of the tube as the light diminished.

This progressive attraction was sometimes very great. For example, in two experiments only 25 per cent of the copepods were positive at the beginning, and nearly 90 per cent at the end. The exceptional experiment was the only one employing so low a rate of decrease as 10 per cent. It is possible that such changes are too slow to be perceptible to the animals (below threshold).

It was thought desirable to determine the effect of increasing light intensity, using direct sunlight, so that a very high intensity range would be available. The experiment was conducted in the open behind the Oceanographic Institution. An inverted bell-jar was used as in the darkroom, with the experimental tube placed inside it, standing vertically. Since it was here impossible to circulate cooled water through the bell-jar, it was simply refilled with cold sea water whenever the temperature rose. The stand holding the bell-jar was completely covered with black tar-paper. A small aperture cut in the top allowed a beam of sunlight to fall on the top of the experimental tube. On one side of the stand, the tar-paper formed a moveable flap which could be lifted, and the necessary counts made. Four opal diffusing discs were placed over the aperture to reduce the light; these were removed at intervals. In the first experiment, they were removed one at a time, in the second two at a time, and in the last experiment all four were removed together. Each disc alone transmits 25 per cent of the light falling upon it. The beam of sunlight was directed on to the aperture above the tube by means of a simple plane mirror which could be turned as the sun changed its elevation. The light intensity was measured by means of a Weston Photronic Cell.

TABLE V

Reactions to direct sunlight

Time	No. of Opals	Relative Sunlight	Approximate Int. in Tube (f.c.)	(Top) I	II	III	(Bottom) IV
		per cent					
12 Noon	4	100	1,080				
12:30 P.M.	4	100	1,080	11	1	4	3
1:00 P.M.	3	98	1,400	11	1	3	3
1:30 P.M.	2	93.6	2,000	12	1	1	4
2:00 P.M.	1	88	2,640	19	–	–	1
2:30 P.M.	0	79	9,470	11	2	1	5
3:00 P.M.	0	73	8,760	11	–	1	5
3:30 P.M.	4	59	636	12	–	3	3
4:00 P.M.	2	43.6	935	5	2	6	4
4:45 P.M.	0	27	3,240	7	1	3	7

The results of the experiment (Table V) show that when the light had increased from about 1,000 to about 9,000 f.c. over a period of two hours, the animals were at all times strongly photopositive. However, increases starting at lower intensities resulted in a majority of the animals in the lower half of each tube. Is there also a negative geotropism which becomes stronger with increase in light intensity? Certainly the results indicate that mere rate and direction of change of light alone cannot account completely for the movements of *Centropages typicus.*

Thus experiments were next carried out in order to test the possibility that the copepods might react to gravity, and that the above results were only partially due to phototropic responses.

The experimental tubes were placed vertically in the bell-jars in the normal way. The animals were then left in darkness, and counts made later with the red lamp. For example, the tubes were left for 1½ hours in darkness and subsequent counts gave the following results (Tubes *A* and *B* were treated identically to furnish checks on each other):

	(Top) I	II	III	(Bottom) IV
Tube *A*...............	14	4	1	–
Tube *B*...............	6	4	–	8

The tubes were then reversed vertically end for end. After one-half hour the following results were obtained:

	(Top) I	II	III	(Bottom) IV
Tube *A*...............	12	5	3	–
Tube *B*...............	12	3	–	3

The tubes were again reversed. After one-half hour the following results were obtained:

	(Top) I	II	III	(Bottom) IV
Tube *A*...............	11	3	1	2
Tube *B*...............	15	1	–	3

The above results clearly show that the animals are on the whole negatively geotropic in darkness. Careful observation showed that the animals sink rapidly if they cease swimming. Hence actual effort was necessary for them to remain at the tops of the tubes, and the geotropism must then be quite strong.

The relation between geotropism and phototropism was then tested by taking the above animals from darkness and illuminating them from below, with the following results:

	(Top) I	II	III	(Bottom) IV
Tube *A* (15-watt lamp below).............	7	4	3	4
Tube *B* (100-watt lamp below)............	–	1	–	19

The results would indicate that negative geotropism is stronger than positive phototropism when the light is weak, while positive phototropism is overwhelmingly strong when the intensity is high.

Tube *B* was returned to darkness and a count 15 minutes later showed that the majority of animals were in Section I.

These results verified the negative geotropism. A 60-watt lamp was then placed below the tubes and the following results obtained:

	(Top) I	II	III	(Bottom) IV
Tube *B* (60-watt lamp below the tube)......	1	–	–	19

Both tubes were again returned to darkness and a count 45 minutes later again showed a large majority exhibiting negative geotropism. A 25-watt lamp was then placed below the tubes:

	(Top) I	II	III	(Bottom) IV
Tube *B* (25-watt lamp below the tube)......	4	1	3	9

The experiment was repeated. The animals again showed negative geotropism in darkness. With a 25-watt lamp below the tubes the results were as follows:

	(Top) I	II	III	(Bottom) IV
Tube *B* (25-watt lamp below the tube)......	4	1	–	12

Finally it was decided to determine the effect of replacing a low light intensity by a high one, when the geotropism and phototropism were in opposition. It has been shown that after exhibiting negative geotropism in darkness, on exposure to a 25-watt bulb from below the distribution was:

	(Top) I	II	III	(Bottom) IV
Tube *B* (25-watt lamp below)..............	4	1	–	12

This lamp was then replaced by a 100-watt lamp. A count after 15 minutes showed:

	(Top) I	II	III	(Bottom) IV
Tube *B* (100-watt lamp below).............	–	–	–	17

All the above experiments definitely establish that the adult female *Centropages* is primarily negatively geotropic and positively phototropic. When the two are acting in opposition, the positive phototropism becomes progressively stronger as the light intensity increases.

Discussion

It is still a controversial matter how far laboratory experiments of the type conducted are applicable to conditions in nature. Throughout all the experiments, however, it was our aim to avoid "shock" conditions, and the use of surface tow-nettings avoided large changes in light intensity during the collections.

It would seem from the experiments with artificial light, that adult female *Centropages typicus* should be right at the surface during most of the day, since they are strongly positively phototropic to a very wide range of light intensities, and it does not seem that continual decreases are always necessary to cause a majority to remain positive, such as was found to be the case with *Acartia clausi* (Johnson, 1938). However, repulsion does occur to some extent on prolonged exposure to very high intensities, and also in the experiments using direct sunlight (Table V) when the illumination increased through such ranges of low intensities as may occur in the early morning. Hence, after considerable exposure to strong sunlight (about midday in summer) and possibly also when the light is increasing in the early morning, *Centropages* might be expected to be a little lower in the water.

G. L. Clarke (1933) however, found that these copepods have a maximum of about 13 m. during most of the day in the Gulf of Maine. Some hauls made in August, 1935, near Woods Hole, were examined and these in general confirmed this finding, although there were cases when the majority were at the surface. (Clarke also did find, for two stations, the majority at the surface.)

In considering this difference it must be remembered that there are other factors acting in nature. Thus, especially at the surface, turbulence may carry the copepods to somewhat lower depths. Further, the possibility of muscular fatigue must not be overlooked. As has been mentioned, *Centropages* will sink rapidly as soon as it ceases swimming, and thus some will tend to sink below the surface, though positively phototropic. This probably accounts for the observation, that, although using the same intensities, a considerably larger percentage of animals is found in the negative half of the tube in the vertical experiments than in the horizontal ones. It should also be noticed that Clarke did find a secondary maximum of *Centropages* at the surface.

The rise to the surface at night, observed by Clarke and others, is explainable since *Centropages* is always very strongly attracted when the light intensity is diminished. The negative geotropism, evident at least during and just after exposure to darkness, will aid the rise.

Parker (1901) found that female *Labidocera* migrate surfacewards at night due to positive phototropism and negative geotropism, and Dice (1914) considered geotropism the major factor in the migration of *Daphnia*. However, the recent findings of Kikuchi (1938) exemplify the fact that the actual rôle played by geotropism probably varies from species to species.

Since *Centropages* is positively phototropic to very low intensities, the upward migration will presumably continue when the light is exceedingly weak. Further, when the animals have reached the surface, they will tend to remain there during darkness owing to the negative geotropism, and they will not take up a more or less uniform distribution, as Russell has supposed for some planktonic species.

As regards the downward migration in the morning, we were generally unable to demonstrate repulsion with increase in light intensity using electric light in the laboratory. However, in the experiments using direct sunlight, it was shown that increase in intensity at a low intensity range from about 700 to 3,500 f.c., did cause repulsion, and this range of light change might be expected in the early morning. It is possible that exposure to darkness during the night might also tend to render the animals more sensitive to light, but there is the opposing geotropism to consider. This has been shown, however, to be definitely weaker for average light intensities. Further experiments, however, are desirable in this connection.

Although no experiments were conducted to test specifically Esterly's theory of a diurnal rhythm (Esterly, 1917, 1919), it would seem from an examination of our readings at different times of the day that such a rhythm is absent in *Centropages*. Rose considers that a species exhibiting diurnal vertical migration is adapted to a certain optimum light intensity (Rose, 1925). Many investigators have been unable to demonstrate such optima in the laboratory. Esterly, for example, found *Calanus* negative to all intensities used, provided the temperature was above 10° C. Rose believed that if a wide range of intensities was employed in the experiments, the optima would be demonstrable. We therefore used a very wide range in our experiments, but did not find any such optimum for *Centropages*.

Reversal of phototropic signs with absolute intensity of light was also difficult to obtain, though Loeb, Parker, Rose, etc. have demonstrated this for many planktonic species. It should be noted that Clarke also found there was no evidence from his experiments for an optimum light intensity in *Daphnia*. He also found that reversal of phototropic sign could not be brought about by absolute light intensity in this form. (Clarke, 1930 and 1932.)

Various authors have frequently pointed out the complexity of the problem of vertical migration by showing differences in behaviour between different species (e.g. Clarke, 1933), between the sexes of a single species (e.g. Russell, 1928), and between ages of the same sex of one species (e.g. Gardiner, 1933). The observations of the authors of the present paper further illustrate that although the majority of adult female *Centropages typicus* do behave in a similar manner, variation in vertical distribution between individuals may be expected even when they are of the same species, sex and age. This is in agreement with field studies.

Summary

Experiments on phototropism and geotropism in adult female *Centropages typicus* were conducted. The following conclusions were indicated:

A. Experiments with experimental tube horizontal.

1. The copepods are primarily photopositive and constant exposure does not modify this reaction except at very high intensities in the neighborhood of that of bright sunlight (*ca.* 12,000 foot-candles) when a large number exhibited negative phototropism after continual exposure for about an hour.

2. The lowest intensity at which there were always more copepods in the brighter than the darker half of the tube was *ca.* 0.005 f.c.

3. There are two types of individuals. One type, after continuous exposure to light, becomes indifferent. In the other type, the animals are persistently attracted.

4. Decrease in light intensity, at a variety of rates and at a wide range of intensities, always results in increased attraction.

5. Increase in light intensity, at a variety of rates and at a wide range of intensities, has no effect on the behaviour. Only prolonged exposure at high intensities repels the animals.

B. Experiments with experimental tube vertical.

1. With the light from above the animals stay mainly at the top of the tube through a wide range of intensities, a distribution which is probably the result of positive phototropism, negative geotropism, or both.

2. Increases in intensity have no effect on the animals except when sunlight is used. A fair percentage of the animals is then repelled.

3. With the exception of decreases as low as 10 per cent per hour, decreases in intensity result in increased attraction.

4. The animals are strongly negatively geotropic in darkness. When geotropism and phototropism are opposed, the reactions depend upon the intensity of the light.

5. The possible bearing of these conclusions on the vertical distribution and diurnal vertical migration of adult female *Centropages typicus* is discussed.

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EMBRYONIC INDUCTION IN THE ASCIDIA

S. MERYL ROSE

(From the Department of Zoology, Columbia University, and the Marine Biological Laboratory, Woods Hole, Massachusetts)

INTRODUCTION

The Ascidia are grouped with those animals whose early development is termed mosaic. Yet, in the closely related Vertebrata, organs form as the result of interaction between those cells which become the definitive organ in question and neighboring cells, whose descendants take no part in the actual formation of the organ. The independent differentiation of organs in the Ascidia and the dependent differentiation of the same organs in the Vertebrata presents a problem.

It is believed that the Ascidia and the Vertebrata are descendants of a common ancestor which contained organs similar to those now common to both groups. The nerve cord, for example, in the two groups is thought to be homologous. The common ancestor must have had a nerve cord which arose either under the influence of inductors or independently as a mosaic piece. It seems strange that in the course of evolution the vertebrate nerve cord and the ascidian nerve cord could have remained such similar embryonic structures, when their modes of origin were diverging so greatly that the one now forms under the influence of inductors and the other quite independently of inductors. Possibly this difference in mode of development between the two groups is more apparent than real.

The injury experiments of Conklin (1905*b*) established the fact that in *Styela partita* surviving blastomeres do not deviate from their prospective potency by regulating to form more morphological units than they normally form as parts of a whole embryo. Conklin's work further showed that differences in protoplasmic appearance and cleavage peculiarities develop in the uninjured blastomeres just as they would were the blastomeres part of an intact embryo. These differences are numerous enough to allow a careful observer to differentiate the presumptive tissues and organs before the formation of the definitive structures. This fact, I think, is the basis for the belief that the ascidian egg is a mosaic of self-differentiating parts. The isolated parts certainly self-differentiate into what are recognizably distinct presumptive regions, but the question is whether any or all of these isolated presumptive regions are capable of further self-differentiation into embryonic organs.

The problem, then, is to determine whether or not inductive influences are present in the developing ascidian embryo, and, if they are present, which cells release inductors and which structures develop in dependent fashion. The answers have been sought with the aid of isolation and transplantation techniques.

I wish to express deep gratitude to Dr. Barth for his valuable suggestions during the course of this work.

MATERIALS AND METHODS

The animal chosen for these experiments was *Styela partita*. This particular animal was used because its normal embryonic development is comprehensively portrayed and because the mapping of presumptive regions is complete (Conklin, 1905*a*). A further reason for using the egg of *Styela* is that much of the experimental work on early ascidian development with which the present work must be compared was done on this egg.

Fertilized eggs were obtained in two ways. In the early part of this work several animals were cut in two and the eggs and sperm removed from the gonads and ducts in a pipette and mixed in sea water. Only a small percentage of eggs was fertilized. These were recognized by the cap of concentrated yellow pigment which forms after fertilization and were sorted out for use. This method is laborious and leaves little time before the first cleavage occurs in which to prepare the eggs for operations. Fertilized eggs are obtained more easily and quickly from spawning animals. Usually *Styela* spawn in the laboratory some time between 4 and 7 P.M. However, they may be induced to spawn at any time of day or night by subjecting them to light for eleven or twelve hours preceding the desired time of spawning. The animals were kept in running sea water aquaria where the light was controlled with an opaque oil-cloth cover and an electric light. Bulbs of 40 and 150 watts placed directly over the tank and about eighteen inches from the animals were found to be equally effective. As a rule the aquarium was shaded with the oil-cloth during the afternoon and evening and the light turned on at about 10 P.M. The animals then started to spawn the next morning between nine and ten. The time between the spawning of the first animal and the last varied from fifteen minutes to several hours. During the longer spawning periods eggs were collected for use several times. The same group of animals could be induced to shed clouds of sperm and eggs on four or five successive days, by controlling the illumination.

There is something released into the water by the spawning animals which induces others to spawn, provided that the latter have had almost the necessary eleven or twelve hours of light. This knowledge was used occasionally in causing the animals of one tank to spawn several hours before the expected time by adding some water from another tank in which spawning had ceased shortly before.

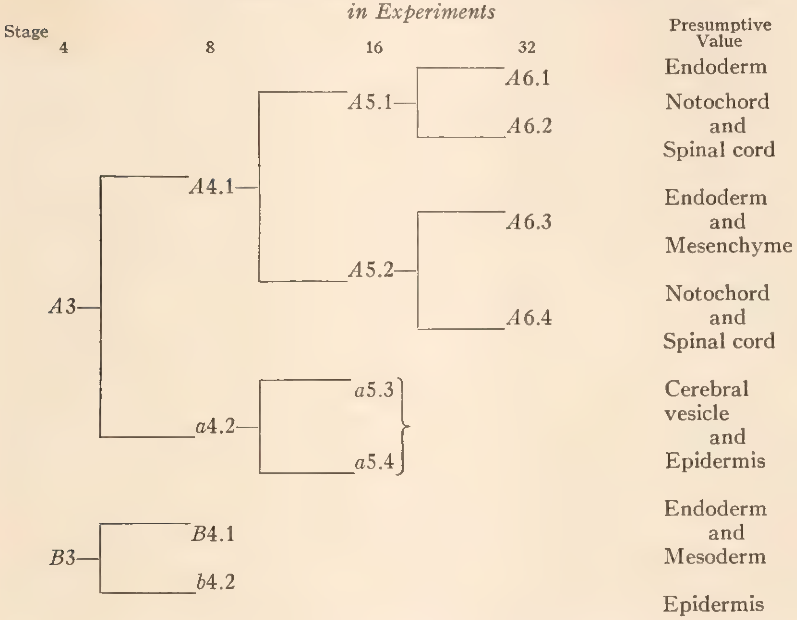
Eggs were carried in small pipettes through eight washes of pasteurized sea water. The water had been heated to 70° C. and maintained at that temperature for five or ten minutes. After cooling it was aerated by shaking and used immediately or kept in the refrigerator overnight until shortly before use the next day. The operating dishes were 20 mm. Stender dishes. These were flamed with a Bunsen burner each time before use and a hot 1.5 per cent agar solution in sea water was permitted to cool and solidify upon the bottom of each dish. The smooth agar surface prevented the eggs from adhering to the glass. The operating solution was 0.4 per cent 0.1N HCl in pasteurized sea water, which changes the pH to approximately 7.6. The sea water was slightly acidified because most of the eggs, after removal from the membranes in ordinary sea water, cleaved abnormally and often cleavage furrows disappeared although nuclear divisions continued, very much as is the case when *Arbacia* eggs are treated with alkaline or acid sea water (Smith and Clowes, 1924). Acid rather than base was tried because Child (1927) had found more normal development of *Corella willmeriana* embryos outside of the atrium when the CO_2 tension was increased. Child found the pH of the atrium to be approximately 7.4. In the acidified sea water injury from manipulation was much less frequent. The eggs seemed more viable and, without membranes, could develop into normal tadpoles not distinguishable from those grown within the protective membranes. Pasteurized sea water and semi-sterile precautions with operating dishes and instruments were employed because survival with good differentiation was increased from about 10 per cent to over 90 per cent by so doing. Instruments were dipped in alcohol between operations and the pipette shaft flamed each time after use.

Membranes were removed from the eggs in operating dishes with the aid of fine watchmakers' forceps. The denuded eggs were then transferred to other operating dishes in finely tipped pipettes. Blastomeres were separated with Spemann glass needles. Transplantations were accomplished by simply bringing one cell or group of cells to rest upon another group with which combination was desired. The cells of the cleavage stages are quite sticky and adhere readily.

Embryos were fixed in Bouin's fluid. After fixation they were transferred to a 1.2 per cent agar solution as it was cooling. After solidification of the agar, blocks containing the embryos were cut out and passed through the alcohols. The 95 per cent alcohol through which the blocks were passed during dehydration contained some water-soluble eosin. The blocks and the embryos were stained enough so that they might be seen more easily during clearing and imbedding. This is a modification of a technique employed by Dalcq (1932) for the manipulation of ascidian embryos. Sections of seven micra were cut and then stained a few minutes in Heidenhain's haematoxylin at 45° C. after a previous mordanting of twenty minutes in 4 per cent ferric alum. Further staining for three minutes in 1 per cent light green after treatment in 0.5 per cent phosphotungstic acid for five minutes was sufficient to stain the yolk material. A 0.5 per cent solution of eosin in slightly acidified 95 per cent alcohol counterstained sufficiently in thirty seconds.

TABLE I

Presumptive Value and Cell Lineage of Cells Used in Experiments


Stage
4
8
16
32
Presumptive Value
A6.1
Endoderm
A5.1
A6.2
Notochord and Spinal cord
A4.1
A6.3
Endoderm and Mesenchyme
A5.2
A3
A6.4
Notochord and Spinal cord
a5.3
a4.2
a5.4
Cerebral vesicle and Epidermis
B4.1
Endoderm and Mesoderm
B3
b4.2
Epidermis

The photomicrographs were taken through an oil immersion lens. I wish to thank Mr. J. Godrich for his part in the preparation of the photographs and plates.

EXPERIMENTAL SECTION

In Table I the presumptive value and cell lineage of the cells used in the experiments to be described are given. The presumptive value and cell lineage were worked out by Conklin (1905*a*). The relative positions of the cells described in Table I may be seen in Figs. 1–4.

In both the table and figures the cell notations are given for only one side of the embryo, since the cleavage pattern is bilaterally symmetrical. When reference is made to corresponding cells of both sides of the embryo, the figure 2 is placed before the cell lineage notation. A figure greater than 2 indicates that corresponding cells of more than one embryo have been used.

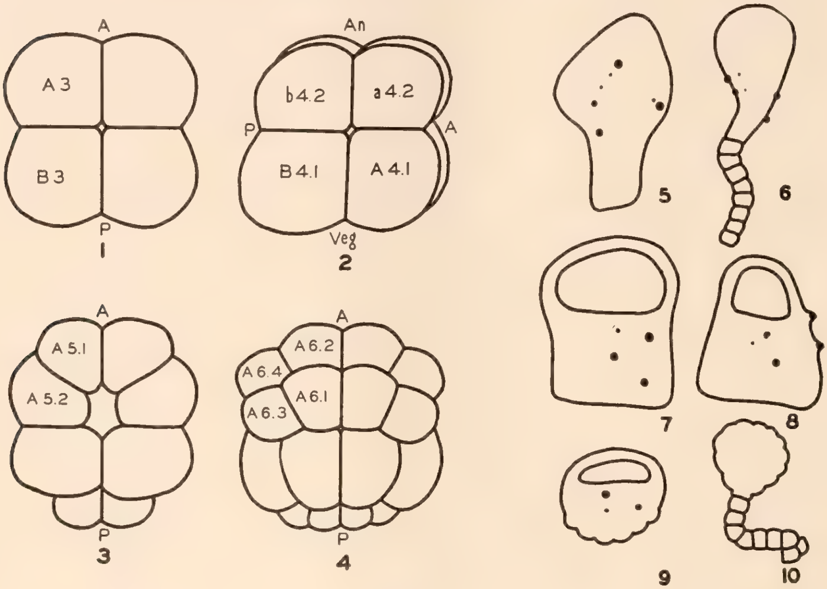


PLATE I

Abbreviations: *A*, anterior; *An* animal pole; *P*, posterior; *Veg*, vegetal pole. The cell lineage notations may be understood by referring to Table I.

FIG. 1. A vegetal view of a four-cell stage.

FIG. 2. A right side view of an eight-cell stage.

FIG. 3. A vegetal view of the eight vegetal cells of a sixteen-cell stage.

FIG. 4. A vegetal view of the sixteen vegetal cells of a thirty-two cell stage.

FIGS. 5–8. Surface views of anterior half, 2*A*3, embryos. Supernumerary pigment spots are present in all and a bare notochord is shown in Fig. 6.

FIG. 9. A surface view of an anterior quarter embryo, 1*A*3.

FIG. 10. A surface view of an anterior vegetal quarter embryo, 2*A*4.1. A notochord is present.

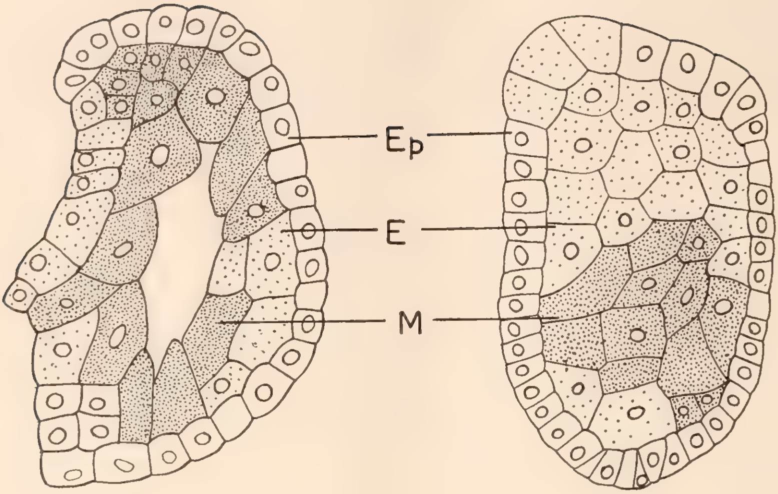
The early cleavages allow an experimental isolation of the presumptive regions and combinations of various regions in order that the normal interactions may be ascertained.

Comparison of Anterior and Posterior Half Embryos

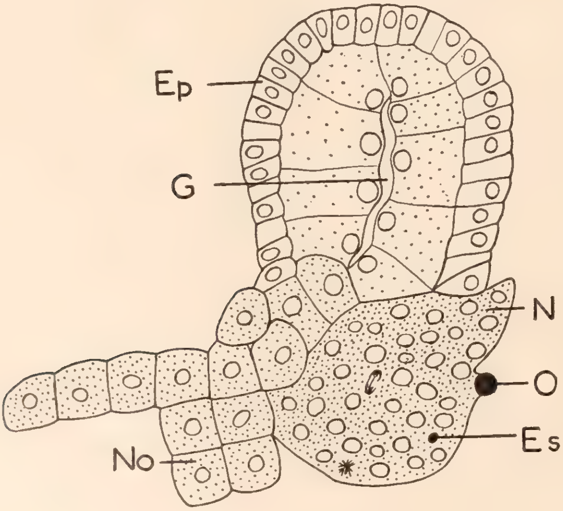
Separation of the yellow, *B*3, and gray cells, *A*3, in the four-cell stage serves to test to what extent the two may differentiate independently of each other. Both parts have been shown, by Chabry (1887) in *Ascidiella*, and by Conklin (1905*b*) in *Styela*, to undergo partial cleavage and to gastrulate. The anterior or gray cells may form a notochord, neural structures including the pigmented sensory cells (otolith and eye-spot), and endoderm which in some cases becomes arranged in the form of a gut with lumen. Figure 13 is a drawing of a section of an anterior half embryo showing the above-mentioned features. The high degree of differentiation of the anterior partial embryos is in agreement with the results of Chabry and Conklin.

A peculiarity shown by approximately half of these anterior half embryos, grown from 2*A*3 cells, is the presence of more than the normal two sensory spots. Figures 5–8 show surface sketches of four such embryos. Figure 9 shows an anterior quarter embryo grown from 1*A*3. The greatest number of pigment spots observed in the anterior half embryos was nine. Often pigment formed in cells widely separated, sometimes on opposite sides of the embryo. In many of the anterior embryos the neural plate did not fold over to form a cerebral vesicle, but, instead, remained on the surface of the embryo. This was usually the case when supernumerary sensory spots were formed. Figures 15 and 16 are adjoining sections of a 2*A*3 embryo which has an infolded embryonic nervous system. One of the pigment spots is external and three are internal. One of the internal sensory cells was cut in such a way as to be included in both sections. It is readily seen that more pigment is produced by anterior half embryos than would be produced by such cells when part of a whole embryo. The presence of extra sensory cells has also been observed in unoperated embryos. Here, however, their occurrence is rare, and never more than four have been seen in one embryo. The phenomenon of supernumerary pigment spots will be further discussed below.

Contrasted with the rather complete differentiation of the anterior half embryo is the unorganized condition of the posterior half. Gastrulation occurs and the embryos survive past the time when the controls become swimming tadpoles, but the presumptive muscle cells remain large and almost round, never elongating nor taking on the fibrous appearance of muscle cells. Figure 11 is a drawing of a section of a posterior half embryo showing the absence of differentiation. Chabry (1887) cultured posterior half embryos of *Ascidiella* and found poorer development of posterior than of anterior halves. No mention was made of muscles. Conklin (1905*a*, p. 52, footnote), employing the convention of calling a cell a "muscle cell" if, in normal development, it would give rise to nothing but muscle, designated these undifferentiated cells of the partial embryos, muscle cells. Since this work is



11 12



13

PLATE II

Abbreviations: *Es*, eye-spot; *Ep*, epidermis; *E*, endoderm; *G*, gut; *M*, mesoderm; *N*, neural tissue; *No*, notochord; *O*, otolith.

FIG. 11. A drawing of a section of a posterior half embryo, 2*B*3, containing epidermis, mesoderm and endoderm.

FIG. 12. A drawing of a section of a posterior vegetal plus anterior animal embryo, 2*B*4.1 + 2*a*4.2, containing epidermis, mesoderm and endoderm.

FIG. 13. A drawing of a section of an anterior half embryo, 2*A*3, containing epidermis, gut, notochord and neural tissue with otolith and eye-spot.

concerned with the problem of differentiation, such cells are considered as presumptive, and the term "muscle cell" is reserved for those cells which attain the stage of differentiation found in contractile tissue and acquire myofibrillae.

The isolation and study of twenty-seven anterior and posterior embryos have shown that the gray cells, the *A*3, contain within themselves the ability to self-differentiate, whereas the yellow cells, the *B*3, lack something which would enable them to differentiate.

Animal and Vegetal Embryos

It is possible to observe the development of presumptive epidermal and cerebral vesicle cells isolated from mesodermal and endodermal cells by separating the animal from the vegetal blastomeres in the eight-cell stage. In forty-five such cases there was never evidence of neural differentiation in either the animal or vegetal half. Nothing like a neural tube formed, nor did sensory cells develop. The picture one obtains from sections of partial embryos of the animal region is one of undifferentiated cells showing no cerebral vesicle (Figs. 17–19). Instead of a row of epidermal cells surrounding a vesicle of neural tissue bearing pigment spots in two of the cells, the isolated animal embryos show nothing but a group of closely packed similar cells usually arranged about a cavity. This cavity formed between the dividing cells before the time of formation of neural tissue at the time when control embryos were gastrulae. Some of the animal embryos have a wrinkled appearance and instead of a single cavity, contain several.

Tung (1934), performing the same operation in *Ascidiella scabra*, obtained animal embryos, some of which he believed contained neural tissue. These embryos showed folds or depressions, the cells of which stained more heavily with eosin than did the other cells, or contained a few cells grouped together making a small tube. Since the cerebral vesicle in normal embryos stains more readily with eosin than do the other tissues, and since neural structures arise through a folding process, Tung thought his animal embryos possessed neural tissue. The stain criterion may be reasonably doubted. Tung shows that the presumptive neural cells in the gastrula stage are already eosinophil. At this time the cells are undifferentiated. It seems inadvisable, therefore, to use the eosinophil nature of the cells as a criterion of neural differentiation.

Conklin (1905*b*) also recognized neural tissue in isolated anterior animal cells, but used different criteria. His criteria were that the cells in question in the living condition were very clear cells, as are the neural plate cells of a whole embryo, and, further, that their cell lineage and size were the same as the neural plate cells of a whole embryo. These criteria of neural differentiation seem valid for only the very beginning of differentiation of neural cells. A better criterion would be the formation of a structure more like the normal cerebral vesicle, a vesicle bearing an otolith or an eye-spot. Such has never been recorded from isolated animal cells. Never in past work (Chabry, Conklin, Tung), nor in the present work have sensory structures been seen to develop in isolated animal cells. It seems, then, that there must be some factor extrinsic to the presumptive brain cells which enables them to differentiate.

Isolation of the vegetal quartet of blastomeres, 2*A*4.1 + 2*B*4.1, in the eight-cell stage should test whether vegetal cells are able to self-differentiate. Few vegetal half embryos survived until the time when differentiated structures might be expected. The great majority continued to cleave until gastrulation time. Then the embryos became loosely adhering masses of cells which soon disintegrated. One, however, remained intact long enough to produce a differentiated notochord. The vegetal embryos of *Ascidiella* produced by Tung (1934) show a higher degree of differentiation.

Notochordal cells have also been seen to form in quarter embryos derived from the anterior vegetal cells alone, the 2*A*4.1. Figure 9 is a surface view of such an embryo showing the bare notochord.

Abbreviations: *A*, cup of animal cells; *M*, myofibrillae; *No*, notochord; *Ot*, otolith; *V*, plug of vegetal cells.

FIG. 14. A section through the cerebral vesicle of an unoperated tadpole, showing the size of the larger pigment spot, the otolith.

FIG. 15. A section through the pigment spot region of an anterior half embryo, 2*A*3.

FIG. 16. An adjoining section of the same embryo shown in Fig. 15.

FIG. 17. A section through an animal half embryo, 2*a*4.2 + 2*b*4.2, showing several cavities.

FIG. 18. A section through an animal half embryo, showing the unorganized nature of the embryo.

FIG. 19. A section through an anterior animal quarter embryo, 2*a*4.2, showing epidermal vesicle formation.

FIG. 20. A section of a 2*A*4.1 + 2*b*4.2 embryo through the induced cerebral vesicle and otolith.

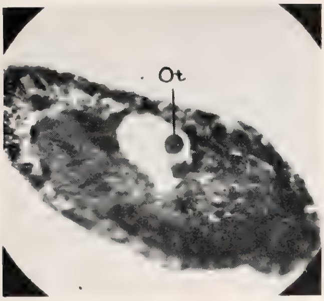
FIG. 21. A section through a 2*A*4.1 + 2*b*4.2 embryo, showing the induced pigment spot.

FIG. 22. A section through a 2*a*4.2 + 1*A*5.2 embryo. The otolith is attached to the cup of cells which arose from the 2*a*4.2. Inserted in the concavity of the cup may be seen the plug of cells derived from the *A*5.2 cell.

FIG. 23. The section passes through a cerebral vesicle containing a typical otolith in a 2*a*4.2 + 2*A*5.1 combination.

FIG. 24. A tail section of an unoperated embryo. The central notochord is flanked by the rows of dark myofibrillae.

FIG. 25. A tail section of a posterior three-quarter embryo, showing the notochord flanked by rows of dark myofibrillae.



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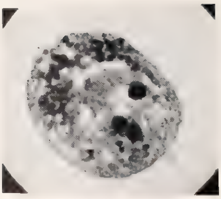

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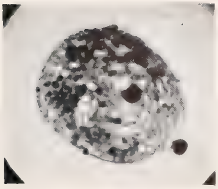

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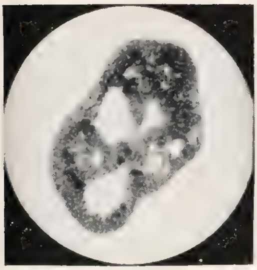

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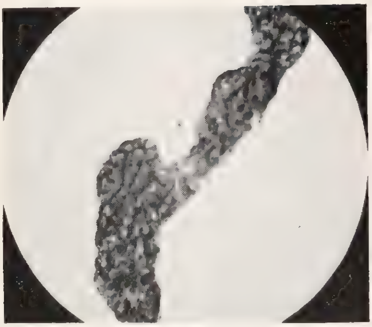

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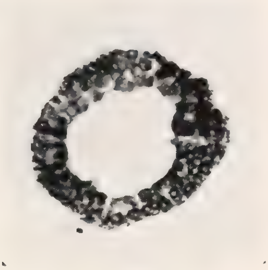

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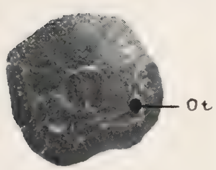

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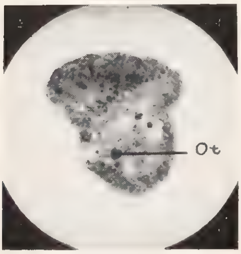

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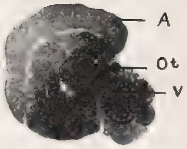

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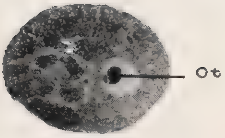

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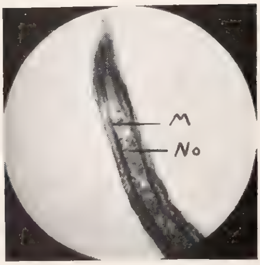

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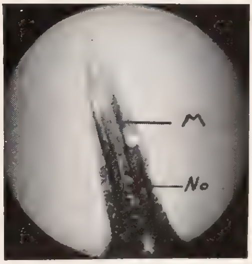

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PLATE III

The isolation experiments seem to indicate that factors or inductors necessary for the differentiation of other parts of the embryo are located in the anterior vegetal region. The evidence for this is the absence of differentiation in embryos which lack this region and the higher degree of differentiation of embryos which contain the anterior vegetal material.

Transplantations

A more striking and positive demonstration of an inductor is obtained when the inductor region in combination with cells incapable of self-differentiation causes those cells to form a structure which neither the inducing nor the reacting cells would form in normal development. Combinations of $2A4.1 + 2b4.2$ from the eight-cell stage have led to the development of partial embryos possessing cerebral vesicles and sensory cells. Figures 20 and 21 are sections of two such embryos through the otolith region. The number of $A4.1 + b4.2$ combinations which produced embryos containing pigmented sensory cells was fifteen out of forty-six. In these embryos presumptive epidermis cells have replaced presumptive cerebral vesicle cells, and, in combination with inductor, have formed cerebral vesicles and the pigmented sensory cells.

An attempt to determine the extent of the cerebral vesicle inductor in the anterior vegetal quadrant has been made. The $A5.1$ and the $A5.2$ cells of the sixteen-cell stage and the $A6.1$ and $A6.3$ and the $A6.2$ and $A6.4$ cells of the thirty-two cell stage have been combined with animal cells of the eight-cell stage. Both the $A5.1$ cells and the $A5.2$ cells have induced cerebral vesicles and sensory cells. Figure 23 shows a well-formed otolith in a cerebral vesicle. This embryo arose from a $2a4.2 + 2A5.1$ combination. Figure 22 is a section through a $2a4.2 + 1A5.2$ embryo. It is of interest because it shows that the otolith formed from an animal cell which was in direct contact with $A5.2$ derivatives. The animal cells are seen in the form of a cup with a plug of vegetal cells protruding from the concavity of the cup. A few combinations were made in which one member of the pair was stained with Nile Blue Sulphate. In five instances gastrulation was incomplete and in these the sensory cells formed on the surface at the boundary between the stained and unstained portions. The proximity of the inducing vegetal cells and the reacting animal cells suggests a direct transfer of inducing substance from the vegetal to the animal cells.

The extent of the inductor in the thirty-two cell stage is less clear. One thirty-two cell embryo from which the $2A6.2 + 2A6.4$ cells were removed produced a sensory pigment cell. This was the only operation of this type performed. In this embryo the only anterior vegetal cells present were derivatives of the $2A6.1$ and $2A6.3$ cells, presumptive for endoderm and mesenchyme. The presumptive notochord and spinal cord cells were removed. A few other operations testing for the presence of neural inductor in the $A6.1$ and $A6.3$ derivatives were performed late in the operating season during September when sensory pigment was not forming in the operated embryos, or even in a number of the control embryos. The combination was $A6.1 + A6.3 + 2a4.2$. Of four successful combinations, two showed evidence of neural invagination. One of these two contained a solid internal rod of neural type cells. Of four embryos resulting from $A6.2 + A6.4 + 2a4.2$ combinations, none showed any evidence of neural invagination. The negative cases are so few here and the criteria of neural differentiation so limited that our knowledge of the neural inducing ability of the $A6.2$ and $A6.4$ cells remains uncertain. The $A6.1$ and $A6.3$ cells in the whole embryo give rise to the endodermal cells which directly underlie the cerebral vesicle. It is probably they, in normal development, which induce the cerebral vesicle.

Twenty combinations of $2B4.1 + 2a4.2$ gave no evidence of neural differentiation (Fig. 12). The embryos are very similar in appearance to posterior half embryos, $2B3$ (Fig. 11). This result indicates that the neural inductor is limited to the anterior vegetal region and does not spread over the entire vegetal region.

The extrinsic factors functioning in muscle differentiation will be described in a future paper. At present, it may be said that the presumptive muscle cells, when they are part of a posterior half embryo, do not self-differentiate (Fig. 11). Neither do they differentiate when combined with anterior animal material, $2B4.1 + 2a4.2$ (Fig. 12). Functional tail muscles do form, however, in posterior three-quarter embryos. The operations were performed in the thirty-two cell stage when the $2A5.1 + 2a5.3$ cells were removed, leaving the $2a5.4$ and $2A5.2$ cells in combination with the posterior half of the embryo. Figure 11 is a photograph of a section of an unoperated embryo's tail, and Fig. 12 is a similar section of a tail of a posterior three-quarter embryo. Myofibrillae may be seen in both sections.

Potency to Respond to Cerebral Inductor

The relative potency to respond to the cerebral inductor has been found to differ in various parts of the embryo. Table II is a summary of the data. The normal number of sensory pigmented cells found in the cerebral vesicle of *Styela* is two. Rarely four appear. Blastomere combinations which are predominantly of anterior materials regularly produce sensory cells. These cells, which form in normal development in the brain, may be considered to be evidence of the presence of neural differentiation, even though in many cases a neural tube has not formed. The number of sensory cells which developed in the anterior embryos was often greater than two and the amount of pigment was greater than in whole tadpoles. The numbers of pigment cells range from one to nine, the average being 3.8 for the anterior half embryos. The *a*4.2 + *A*5.1 or *A*5.2 combinations also regularly produce neural tissue. Supernumerary sensory cells may also appear in

TABLE II

Reaction to Cerebral Inductor

Combination	Presumptive Value	No. with Sensory Cells	No. without Sensory Cells	No. of Sensory Cells
2*A*3	*Ep, CV, Not, SC, G, Mes.*	22	2	1–9
2*a*4.2+1*A*5.1	*Ep, CV, Not, SC, G.*	2	0	1–4
2*a*4.2+2*A*5.1		4	0	1–4
2*a*4.2+2*A*5.2	*Ep, CV, Not, SC, G, Mes.*	5	1	1–4
2*a*4.2+1*A*5.2		1	0	1
2*a*4.2+2*b*4.2+2*A*5.1	*Ep, CV, Not, SC, G.*	2	3	1–2
2*a*4.2+2*b*4.2+1*A*5.1		1	0	2
2*a*4.2+2*b*4.2+2*A*5.2	*Ep, CV, Not, SC, G, Mes.*	3	1	1–4
2*a*4.2+2*b*4.2+1*A*5.2		1	2	2
2*A*3+1*B*4.1	*Ep, CV, Not, SC, G, Mes, Mus.*	3	2	2–3
1*b*4.2+2*A*4.1	*Ep, Not, SC, G, Mes.*	3	0	1–2
2*b*4.2+2*A*4.1		7	35	1–2
2*b*4.2+1*A*4.1		3	6	1–2
4*b*4.2+2*A*4.1		1	0	2
5*b*4.2+2*A*4.1		1	0	1
2*b*4.2+1*A*5.1	*Ep, Not, SC, G.*	0	1	–
2*b*4.2+2*A*5.1		0	3	–
2*b*4.2+1*A*5.2	*Ep, Not, SC, G, Mes.*	0	1	–
2*b*4.2+2*A*5.2		0	3	–
1*b*4.2+2*A*5.2		1	0	1
2*B*4.1+2*b*4.2+1*A*4.1	*Ep, Not, SC, G, Mes, Mus.*	0	8	–

CV, cerebral vesicle; *Ep*, epidermis; *G*, gut; *Mes*, mesenchyme; *Mus*, muscle; *Not*, notochord; *SC*, spinal cord.

these embryos. The sensory cell production in *a*4.2 + *A*5.1 or *A*5.2 embryos may be contrasted with that of embryos whose animal material comes from the posterior region, *b*4.2 + *A*5.1 or *A*5.2. The *a*4.2 material has responded positively in twelve of thirteen cases, whereas the *b*4.2 material gave a negative response in eight of nine cases. This comparison is of embryos from the same batches of eggs. The response of posterior animal cells is somewhat better when the inductor cells are the *A*4.1 cells of the eight-cell stage. In this case both *A*5.1 and *A*5.2 materials are represented. The positive responses with *b*4.2 + *A*4.1 were fifteen of fifty-six. This is in spite of the fact that most of the *b*4.2 + *A*4.1 operations were performed before the introduction of the semi-sterile technique.

Not only do anterior animal cells respond more often than posterior animal cells to the same inductor, but also the anterior cells produce more sensory structures. Never have the posterior animal cells produced more than two sensory cells. The number is usually one.

A further result obtained from the transplantation experiments is that the addition of posterior cells to combinations which alone would produce neural material decreases the frequency of its appearance. When the *a*4.2 cells alone were in combination with *A*5.1 or *A*5.2, twelve of thirteen embryos contained sensory cells. When *b*4.2 + *a*4.2 cells were host to *A*5.1 or *A*5.2, only seven of thirteen produced sensory cells. Similarly, when the 2*B*4.1 material was added to a 2*b*4.2 + 1*A*4.1 combination, there were no sensory cells produced in eight cases. Alone the 2*b*4.2 + 1*A*4.1 combination had been shown to form sensory cells in three of nine cases.

Although in some of the individual experiments the cases are too few, the combined data seem to allow the following conclusions: (1) Anterior animal cells have greater potency to form cerebral structures than do posterior animal cells. (2) Posterior cells tend to inhibit the formation of sensory structures in embryos containing competent materials.

Discussion

The classical works of Conklin (1905*a*, 1905*b*) on *Styela* demonstrated that early in development there is a segregation of oöplasmic materials. These visible cytoplasmic materials are correlated in normal development with particular embryonic organs or regions. However, some of these substances may be centrifugally displaced and come to lie in foreign organs (Conklin, 1931). In a sense, the segregation of visible oöplasmic materials is differentiation. Further, isolated blastomeres differentiate in respect to cleavage patterns. But differentiation also includes the establishment of the various functional structures. The present work indicates that the anterior vegetal region is necessary for this latter type of differentiation.

The earlier idea that the ascidian egg is a strict mosaic has been altered in recent years. Schmidt (1931) has found that lateral half embryos of *Ciona intestinalis* and *Phallusia mammillata* may sometimes form the normal three adhesive papillae. Cohen and Berrill (1936) obtained some rather normal appearing larvae from lateral half embryos of *Ascidiella aspersa*. They, however, interpreted the regulation as a mechanical regulation of an original mosaic pattern. Recently, von Ubisch (1938) has described a case in which two fused two-cell embryos of *Ascidiella aspersa* regulated to form a single individual.

Dalcq (1932, 1938) has shown that lateral, or animal, or vegetal portions may be removed from the egg before fertilization without resulting depletion of organs in the larvae which develop from the egg. Reverberi (1931) obtained larvae very similar to normal larvae from fragments of fertilized *Ciona* eggs. The results of Dalcq and Reverberi plainly show that the egg is not a determined mosaic before completion of the first cleavage.

Tung (1934) suggested the possibility that adhesive papillae and sensory cells might be dependent upon extrinsic factors, since they did not form in the isolated presumptive cells. The present work indicates that induction of organs is more general in the ascidian embryo. It appears that all cells outside of the anterior vegetal region differentiate dependently. This anterior vegetal region, presumptive for notochord, spinal cord, endoderm and some mesoderm, is similar in function to the corresponding region of the amphibian embryo, the organizer region. It is capable of self-differentiation and supplies necessary developmental factors to other regions. The great difference between amphibian dorsal embryos (Ruud, 1925) and ascidian anterior embryos is that the former regulate and form more than they would as parts of intact embryos, while the latter offer no evidence of regulation.

The recent work of Reverberi (1937) demonstrates that both animal and vegetal materials must be present in egg fragments of *Ciona intestinalis* in order that the sensory cells may differentiate. Reverberi's work and the present work suggest a possible interpretation.

There are fundamental regional differences in the egg. Materials necessary for the differentiation of endoderm and notochord and for the production of inducing substances are in highest concentration in the anterior vegetal region. Materials which react with the cerebral inducing substances, or materials which produce the reacting substances, are more concentrated in the animal region, especially the anterior animal region. The contiguity of original animal and vegetal regions established during gastrulation enables the interaction of anterior vegetal inducing substance or substances and the reacting animal material, which process leads to the differentiation of cerebral vesicle.

CONCLUSIONS

1. Blastomeres from the animal region of the eight-cell stage are incapable of self-differentiation.

2. Posterior blastomeres of the four-cell stage are also unable to self-differentiate.

3. The anterior vegetal blastomeres of the eight-cell stage are capable of self-differentiation.

4. The anterior vegetal region is necessary for the differentiation of other regions.

5. The cerebral inductor is confined to the anterior vegetal region.

6. Presumptive epidermis may form brain under the influence of the inductor.

7. Anterior animal cells have greater potency to form cerebral structures than do posterior animal cells.

8. Posterior cells inhibit the formation of cerebral structures in embryos containing competent materials.

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ANDROGENETIC DEVELOPMENT OF THE EGG OF RANA PIPIENS[1]

K. R. PORTER

(*From the Biological Laboratories, Harvard University and the Department of Biology, Princeton University*)

INTRODUCTION

The aim of the investigator in seeking to initiate androgenetic development is to remove or inactivate the female pronucleus, at the same time leaving undisturbed the male pronucleus (if it is within the egg), the cytoplasm, and conditions essential for activation and first cleavage. To achieve this, especially by mechanical means, it is important that the egg be large, that the position of the egg chromatin be detectable, and that development proceed under laboratory conditions. It is, therefore, not surprising that the amphibian egg has been generally used.

G. Hertwig, in 1911, treated the eggs of *Rana fusca* with radium emanations, then fertilized them, and obtained androgenetic development for what appears to be the first time. Since then a variety of methods have been used to remove or inactivate the egg nucleus (see below). These have been applied to various European species of frogs (G. Hertwig, 1911; P. Hertwig, 1923; Dalcq, 1932) and toads (G. Hertwig, 1913; P. Hertwig, 1923), to various species of *Triton* (P. Hertwig, 1916, 1923; G. Hertwig, 1927; Curry, 1931, 1936; Baltzer, 1933; Baltzer and de Roche, 1936; Hadorn, 1934) and to one American species, *Triturus viridescens* (Kaylor, 1937).

None of these experiments has produced an adult haploid. In general, with androgenetic haploids as with haploids produced by parthenogenesis, gynogenesis and merogany, development ceases after a few days or in some cases a few weeks, is always abnormal, and where it continues to the larval stages produces an animal which is inactive and edematous.

Despite their abnormalities, these haploids offer numerous possibilities for the study of nucleo-cytoplasmic relationships. Indeed, the abnormalities in themselves are not without interest, for an experimental demonstration of their cause should throw considerable light on the problems of differentiation. To be most serviceable as an experimental material, it seems essential that the haploids and the methods by which they are produced should possess certain positive characteristics. Their development should be fairly normal and continue to an advanced stage of differentiation; the peculiarities of haploid development should be uniformly displayed by all animals; the haploid nuclear condition should remain unchanged; and the operative technique should be simple, effective, and capable of producing relatively large numbers. Haploids produced from eggs of various species of amphibia and by a variety of methods have satisfied these criteria to varying degrees, in no case perfectly. In view of this fact it is important to experiment further with new materials and methods.

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[1] Part of data previously presented in thesis submitted to the faculty of Harvard University in partial fulfilment of the requirements for the degree of Doctor of Philosophy, June, 1938; part of data from experiments performed during tenure of National Research Fellowship at Princeton University.

The report which follows presents the results of such experiments. An effective technique for the removal of the egg chromatin from the egg of *Rana pipiens* is described; the development which results from these operated eggs is described and compared with the normal diploid; it is shown that the great majority of these animals develop as haploids; and certain cytological observations are presented which are of possible importance in explaining the abnormalities of haploid development.

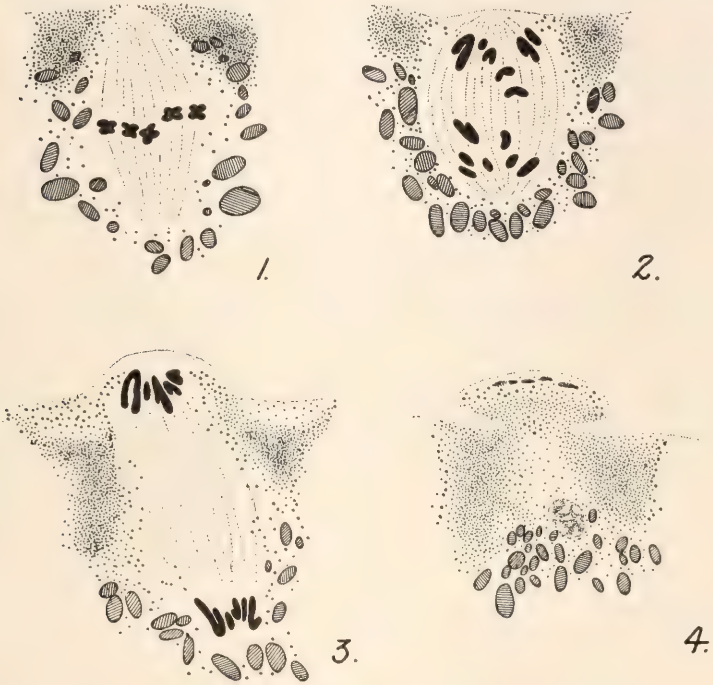
I should like to express my sincere gratitude to Professor Leigh Hoadley for his aid and advice during early investigations of this material. I am also indebted to Professor G. Fankhauser for valued suggestions in more recent studies.

Materials and Methods

The eggs of the frog, *Rana pipiens*, secured from the state of Vermont were used in these experiments. Ovulation was induced by injecting water extracts of the anterior lobe of the frog pituitary following in general the method described by Rugh (1934). Such eggs when inseminated usually give a high percentage of fertilization and since the development which follows is perfectly normal there is little reason for considering the eggs so obtained as inadequate for experimental purposes.

The operation, which results in the removal of the maternal chromatin, is simple and effective. Since it is in part original to these investigations and since its successful application depends on an understanding of events taking place within the egg, a rather complete description follows.

At the time of insemination the egg of *R. pipiens* has undergone the first maturation division and the second division is in metaphase awaiting the entrance of the sperm before continuing in the production of the second polar body and the female pronucleus. Sections through the egg in this stage of maturation reveal the relation of the spindle to the egg surface. (Fig. 1). It is to be noted that it lies close to the surface and is almost completely covered over by pigment granules. As the second maturation division proceeds this relationship is altered.



FIGS. 1–4. Semi-diagrammatic representations of four stages in second polar body formation of *R. pipiens* eggs. Drawings were made with camera lucida and give exact distribution of pigment granules, yolk platelets and chromosomes, only part of which are shown. Selected from considerable material sectioned at 10μ. (Eggs inseminated and kept at 12° C.) 1125 ×.

Fig. 1. Division spindle as in egg at time of insemination.

Fig. 2. Anaphase of maturation division. Stage at which spindle can be seen from exterior of egg as small black dot. Egg fixed 35 minutes after insemination.

Fig. 3. Early telophase. Egg fixed 50 minutes after insemination.

Fig. 4. Polar body just forming. Egg fixed 56 minutes after insemination.

Between 20 and 35 minutes after insemination the metaphase gives way to anaphase and the pigment granules directly over the division figure become widely dispersed (Fig. 2). If, at this time, the egg is observed from the exterior under strong illumination and a magnification of more than 25 or 30 diameters the location of the maturation spindle can be detected as a small black dot. This appearance is doubtless due to the absence of light-reflecting pigment granules over the spindle (Figs. 2 and 3). Many of these so-called black dots have been watched and in all cases they have been observed to disappear gradually (between 35 and 45 minutes after insemination) and to be replaced by the small second polar body (Figs. 3 and 4).

The removal of the egg chromatin is accomplished by means of a glass needle possessing a very fine but rigid point. While the location of the maturation spindle is apparent the point of the needle is inserted through the jelly capsule and into the cortex of the egg to one side of and diagonally beneath the spindle (Fig. 5). A slight upward motion of the needle then produces a small exovate which contains the spindle and consequently all of the maternal chromosomes (Fig. 6). When the operation is performed slowly and carefully the small pellucid spindle can occasionally be seen in the yolky cytoplasm which comes out with the needle. Thus the egg is left otherwise intact with only the male chromatin present to influence the development which follows. The exovate which forms outside the vitelline membrane is soon completely detached from the egg and generally no mark remains on the embryo to mark the place of exovate origin and former attachment.

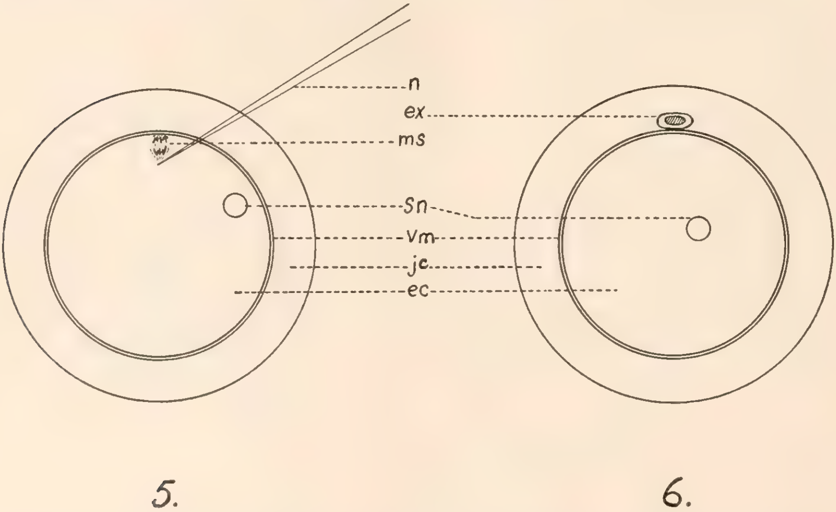


FIG. 5. Diagram of operative procedure. *n*, needle; *ms*, maturation spindle; *sn*, sperm nucleus; *vm*, vitelline membrane; *jc*, jelly capsule; *ec*, egg cytoplasm.

FIG. 6. Diagram of egg and exovate after operation. *ex*, exovate; others as in Fig. 5.

The usefulness and value of such an operation are in part determined by the ease with which it can be executed and therefore the number of eggs which can be treated in a short length of time. Within the 10 to 15 minutes during which the maturation spindles on a group of eggs are apparent it is possible to operate on 25 or 30 eggs and exercise considerable care in so doing. If the eggs are inseminated in small quantities and at 15-minute intervals this number can be increased several times and sufficient material is made available for quantitative studies of a physiological as well as morphological character.

The loss of the small amount of egg cytoplasm which forms the exovate appears to have no harmful effect upon later development. Evidence for this statement is drawn from the following sources: (*a*) Experiments have been performed in which small exovates were produced on eggs in the immediate vicinity of, but not including, the maturation spindle. These developed normally as far as could be observed from external appearances and certainly displayed none of the abnormalities characteristic of haploid embryos. (*b*) Occasionally a normal appearing embryo arises from an operated egg (possibly as result of unsuccessful operation). In two cases these have been allowed to develop and have ultimately metamorphosed without showing any notable deficiencies. Therefore, it seems justifiable to conclude that the abnormal characteristics of the animals which result from these operated eggs are due to an altered nucleus rather than to an altered cytoplasm.

Various other methods have been applied to amphibian eggs to bring about androgenesis. The egg chromatin has been rendered inactive by radium emanations and x-rays (G. Hertwig, 1911, 1913, 1927; P. Hertwig, 1916, 1923; Dalcq, 1929, 1932), it has been removed by pricking the egg of *R. esculenta* with heated and unheated needles (Dalcq, 1932), and it has been destroyed with a needle and then withdrawn by a micropipette (Curry, 1931, 1936; Baltzer, 1933; Baltzer and deRoche, 1936; Hadorn, 1934; Kaylor, 1937).

A comparative evaluation of these various methods should be made only by one who has tried them all. Furthermore, for different eggs, different operations may be required. For example, with the egg of *Triturus viridescens* it is necessary to use a micropipette to remove the egg chromatin for an exovate is not formed by merely pricking the egg. Therefore, whatever may be the merits or drawbacks of these various methods, it is necessary in any evaluation to consider them in conjunction with the egg to which they are applied.

Further comment should be given to Dalcq's method of pricking the egg with heated and unheated needles. It is similar to the technique applied in these experiments to the egg of *R. pipiens* but from his description it does not appear that he observed the exact location of the maturation spindle. Instead, he pricked the egg in the lighter region in the centre of the animal pole where the maturation figure is generally, but not always, located. That he did not always remove the egg chromatin, as he himself suggests, is further indicated by the presence of 5 diploid embryos in a group of 22 which developed from operated eggs.

In all experiments to be reported, experimental animals and controls were from the same female, were inseminated simultaneously, were kept under identical conditions of temperature (generally constant to ± 0.05° C.), volume of water per animal, water change, etc. For fixing, a corrosive sublimate, acetic, formalin mixture was generally used. This has been found to be especially valuable for the younger, yolky stages for it has little hardening effect. Harris haematoxylin has been found most serviceable as a general stain. With it the nuclei stain a deep blue and the yolk granules remain a purple, thus permitting some degree of differentiation.

OBSERVATIONS

The Development of Androgenetic Embryos

The description of androgenetic development which follows is taken from observations on several groups of experimental animals. Developmental rates and illustrations (Figs. 7–22), however, refer to one particular group (Exp. 38–1) numbering 52 experimental animals which were raised at 19.4° C. From this group and one other, experimental animals were selected and fixed at 24-hour intervals as recorded in Tables II and III. Controls were simultaneously preserved. In this way material was provided for an examination of internal as well as external morphogenesis. While some variation is shown among the members of a single group, especially in the older stages, there is a majority which show the general features described below.

Observations were normally begun at the time of first cleavage. This may take place anywhere between 2 and 3 hours after insemination depending on the temperature at which the eggs have been inseminated. It is customary for between 90 per cent and 100 per cent of the operated eggs to divide normally and to do so simultaneously with the control eggs (Table I). This behavior, while typical for these androgenetic frog eggs, is not typical for all amphibian eggs. For example, Kaylor (1937) reports that a considerable proportion of his androgenetic *Triturus viridescens* eggs cleaved abnormally or failed to cleave at all and that 90 per cent of those for which cleavage records were available showed a significant delay in the appearance of the first cleavage furrow.

Blastula development of androgenetic *R. pipiens* embryos is quite normal. As the time for gastrulation approaches slight indentations occasionally appear in the animal hemisphere of the blastula. Since these later disappear, do not occur in all of the experimental embryos, and have been noted in the controls, they are not considered a typical abnormality of androgenetic development. Comparative examination of androgenetic and control late blastulae reveals noticeably smaller cells in the former. This observation made from the outside has been verified from sections. Though cell counts have not as yet been made, it seems probable that there are more cells in the androgenetic blastulae and that they have resulted from a more rapid rate of cell division.

Gastrulation begins approximately one hour earlier in the controls than in the experimental animals. This constitutes the first clear indication of a retardation of differentiation. This delay is a distinct characteristic of amphibian androgenetic development and has been reported by other investigators for a variety of species. It is more clearly indicated at the end of 24 hours by a difference in the size of the crescentic blastopore (smaller in the androgenetic embryos). In the great majority of the experimental animals gastrulation proceeds normally and by the end of 48 hours the yolk plug stage is reached (Figs. 7 and 8). The larger yolk plug of the androgenetic embryo provides evidence of retarded development.

TABLE I

Record of first cleavage in several lots of operated eggs.

Exp. Number	Number of Eggs Operated	Number that Cleaved	Percentage Cleavage
36–5	38	38	100
36–10	179	179	100
36–13	278	255	92
37–2	38	38	100
37–4	70	69	98
38–1	52	52	100

Note: Experiments 36–10, 37–2, 37–4, and 38–1, provided the data upon which this report is based.

During the formation of the neural tube on the third day it becomes clearly evident that androgenetic development is not simply normal development, slightly delayed, but is abnormal as well as delayed. For instance, the neural plate of the experimental animal remains approximately one-third shorter than the neural plate of the control; the neural folds stand up less prominently from the body of

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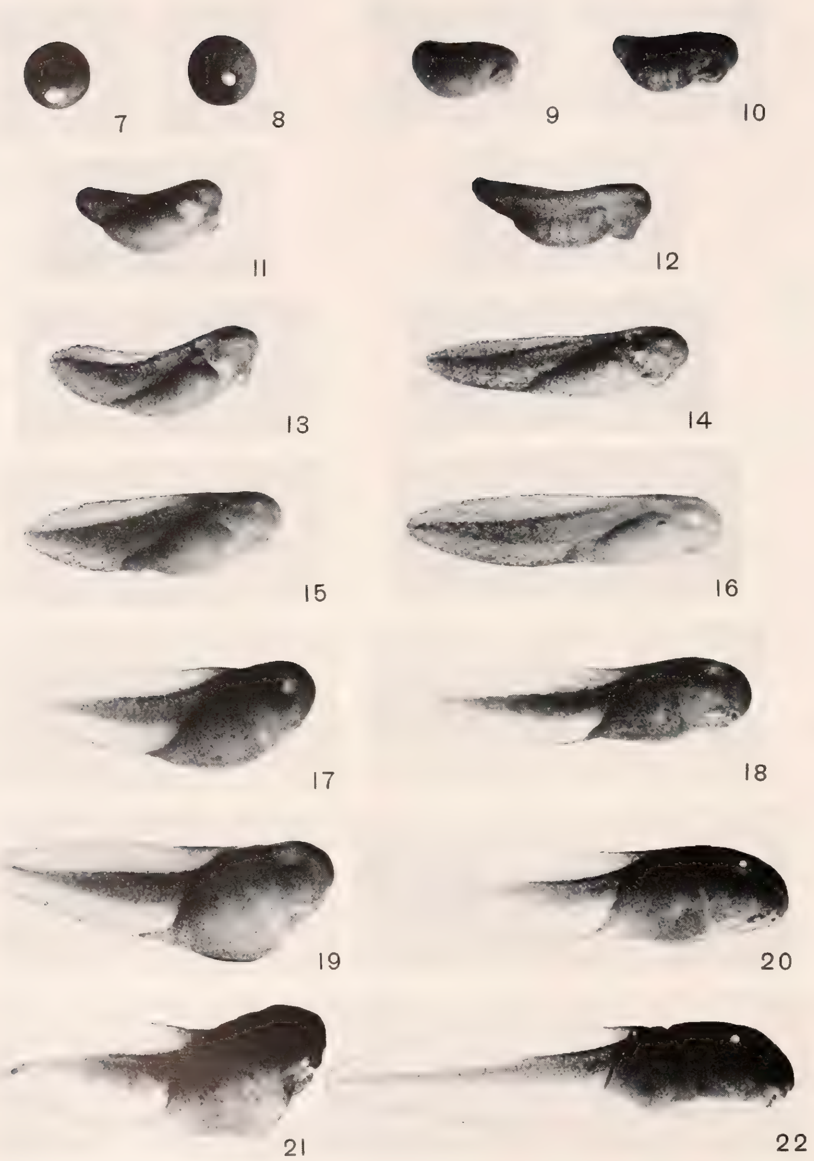


FIGS. 7–22. Photographs of typical androgenetic embryos and normal diploid controls from a group of operated eggs (Exp. 38–1) raised at 19.4° C. Figs. 7, 9, 11, 13, 15, 17, 19, 21 are respectively 2-, 3-, 4-, 5-, 6-, 8-, 10-, 12-day-old androgenetic haploids. Figs. 8, 10, 12, 14, 16, 18, 20, and 22 are respectively 2-, 3-, 4-, 5-, 6-, 8-, 10-, 12-day-old controls. Photographs are of fixed animals. *ca* 5×.

the embryo, and the neural groove is more shallow. There are probably indiscernible abnormalities in gastrulation which contribute to the above and in turn to the more pronounced departures from the normal shown by the older animals. Closure of the neural folds is completed between 2 and 3 hours later than in the controls which indicates an increasing delay in differentiation.

The 3-day-old experimental animal depicts abnormal as well as delayed differentiation (Figs. 9 and 10). The tail-bud is shorter, the abdomen remains abnormally large and round, and the head is smaller and apparently less differentiated. From the third day on development is characteristic of the androgenetic embryos only, and exact stages for stage comparisons with the controls are no longer possible.

The typical 4-day-old experimental animal is smaller than the control, shows a pronounced bend in the back, a shorter and round abdomen, and a head which does not show the normal downward bend or cranial flexure (Figs. 11 and 12). The first indications of gill filaments which appear at this time in the control do not appear in the androgenetic animals until almost a day later.

Certain of these abnormalities persist on the fifth day and are clearly shown in Fig. 13. The 5-day-old control possesses a pulsating heart and a complete gill circulation whereas the experimental animals do not clearly show these features until the end of the sixth day.

In the typical 6-day-old experimental animal (Fig. 15) the back has straightened but in total length the animal is still considerably shorter than the control. It is of interest to note that the head of this animal (Fig. 15) more closely resembles that of the 5-day-old control (Fig. 14) than it does the 6-day-old (Fig. 16). But even in this similarity there are discrepancies as indicated by the position of the olfactory pit. Generally more than one-half of the androgenetic embryos of this age show a pulsating heart and of these fully one-third can be expected to have a fairly normal gill circulation.

It is typical for a few of the 7-day-old animals to become edematous and with each day thereafter the number of edematous animals increases. This condition may become so extreme that not only the body cavity but also the tissue spaces in the head become filled with fluids (Fig. 21). When this extreme is reached death generally ensues. Therefore, if the animals are to be saved, fixing agents are applied. In the group of animals from which this description is illustrated most of the animals were fixed on the ninth and tenth days (Table II).

During the eighth day the operculum grows over whatever gill filaments the animal may happen to have. This operculum development is outstanding in that it takes place at the same time and rate as in the controls whereas other organs may be more than 24 hours delayed.

Table II

A record of fixation and examination for chromosome numbers of animals which developed from 52 operated eggs. (Temp. 19.4° C.)

Age at Time of Fixation	Number Fixed	Classification
1 day	3	3 haploids
2 days	4	2 haploids 1 normal diploid 1 abnormal diploid
3 days	5	3 typical haploids 2 atypical haploids
4 days	2	2 typical haploids
5 days	1	1 haploid
6 days	3	1 typical haploid 2 atypical haploids
7 days	2	2 typical haploids
8 days	1	1 haploid
9 days	11	11 edematous haploids, 5 of which show a few diploid nuclei
10 days	8	8 edematous haploids, 7 of which show a few diploid nuclei
11 days	3	3 edematous haploids, 2 of which show a few diploid nuclei
12 days	6	4 edematous haploids showing a few diploid nuclei 1 edematous haploid with several diploid nuclei 1 diploid-triploid, developed more successfully than the typical haploid
15 days	1	1 haploid-diploid, haploid on one side, diploid on other side.
22 days	2	1 pure diploid of normal structure 1 triploid, appearance of normal diploid

Summary: 46 haploids, 3 diploids, 1 triploid, 1 haploid-diploid, 1 diploid-triploid; 89 per cent of population haploids.

After the eighth day there is slight change in the gross appearance of the experimental animals except that the majority become increasingly edematous (Figs. 19 and 21). Differentiation of some parts continues but a discussion of such differentiation is not essential to this general description. It should be mentioned, however, that in those cases where a circulation is established, at least for a short time, differentiation is more successful and the animal lives over a greater number of days.

The behavior of these animals can scarcely be called normal. Most of the time they are rather inactive and lie on their sides on the bottom of the container. When sufficiently stimulated, however, they will respond by swimming about in undirected circles.

TABLE III

A record of fixation and examination for chromosome numbers of animals which developed from 38 operated eggs. (Temp. 20.1° C.)

Age at Time of Fixation	Number Fixed	Classification
2 days	2	2 typical haploids
3 days	1	1 typical haploid
4 days	2	1 typical haploid 1 slightly atypical haploid
5 days	2	2 typical haploids
6 days	1	1 typical haploid
7 days	10	1 typical haploid 8 edematous haploids 1 very atypical haploid
8 days	4	4 edematous haploids
9 days	11	10 edematous haploids, 5 of which show a few diploid nuclei 1 accidentally destroyed had developed as haploid
10 days	5	1 edematous haploid 2 edematous haploids showing a few diploid nuclei 1 haploid-tetraploid (Fig. 32) 1 died before fixation, had developed as haploid

Summary: 37 haploids, 1 haploid-tetraploid; 97 per cent of population haploids.

Chromosome Numbers and Nuclear and Cell Size

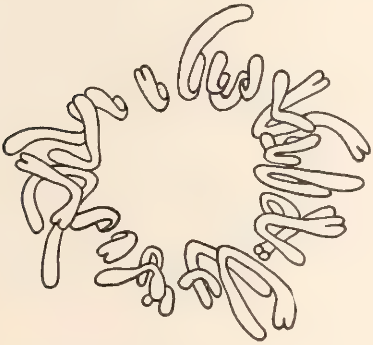
That the embryos which develop from operated eggs are haploids has been indicated, not only by the rather certain removal of the second polar spindle, but also by the abnormalities which they show. For further evidence, however, a cytological examination was made of some part or parts of each animal of two different groups of operated eggs. For the younger animals this evidence was obtained from sections; for the older animals, from tail tips clipped from fixed specimens and made into whole mounts. In the case of each animal one or more metaphase plates were examined in detail to establish the chromosome number, and, in addition to this, a record was kept of the total number of division figures which could be identified as haploid or otherwise by brief examination only. In general, the quality of the preparations permitted the examination of 25 or more (in some cases many more) mitotic figures. The results of these studies are summarized in Tables II and III and additional evidence is shown in Figs. 23 to 27.

It is clearly shown that the vast majority of these operated eggs developed as haploids. For the exceptions there is at the present time no definite explanation. There always remains the possibility that they resulted from unsuccessful operations whereby the egg chromatin remained within the egg. But even if this is the explanation, the results indicate that at its worst the method is about 90 per cent effective. The animals which did not develop as haploids were easily detected for they showed either the characteristics of normal diploids or other characteristics not typical for haploids.

It is of interest to compare these results with those reported by Dalcq (1932) for androgenesis with the egg of *R. esculenta* and by Parmenter (1933) for parthenogenesis with the egg of *R. pipiens* and *R. palustris*. Out of 22 operated eggs in Dalcq's experiments 5 developed as diploids; out of 29 embryos which developed parthenogenetically Parmenter reports 10 pure diploids. These results would lead one to expect a larger number of diploids among these androgenetic *R. pipiens* embryos than have been found. In the case of Dalcq's results, however, the high percentage may be due to a poor localization of pricking and not to any marked instability of the frog haploid nucleus. But failure of operative technique could scarcely account for the large percentage of parthenogentic diploids. Several explanations, which are reviewed by Parmenter, have been suggested. It is possible that a study of very early cleavage stages will provide an explanation for this difference between the results of parthenogenetic and androgenetic experiments.

It has been noted (Tables II and III) that the tail tips of some of the older haploids show a few diploid nuclei. These were identified by their larger size and by the presence of two nucleoli (Fig. 32). Since they occur solely within the tissues of haploids which have more or less reached the end of their development, it would seem that some condition or conditions within these animals are related to their origin. But as to the mechanism of their origin, there is only slight evidence.

In a very few cases monastral divisions of haploid nuclei have been observed. The presence of scattered diploid nuclei in the older stages is not a feature confined solely to these androgenetic haploids.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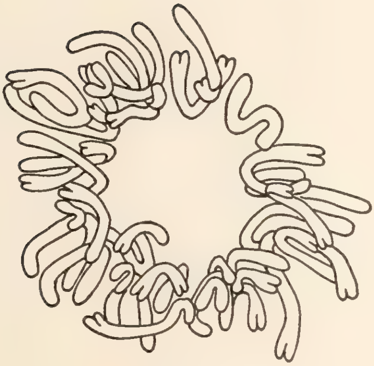
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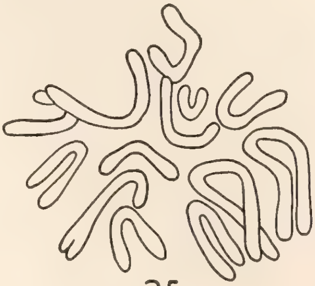
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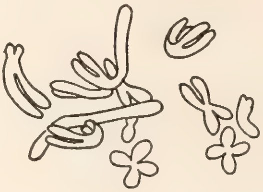
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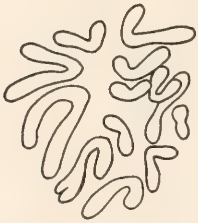
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Figs. 23–27. Camera lucida drawings of mitotic figures. 3250 ×.

Fig. 23. Diploid metaphase from tail epithelium of 15-day-old control.

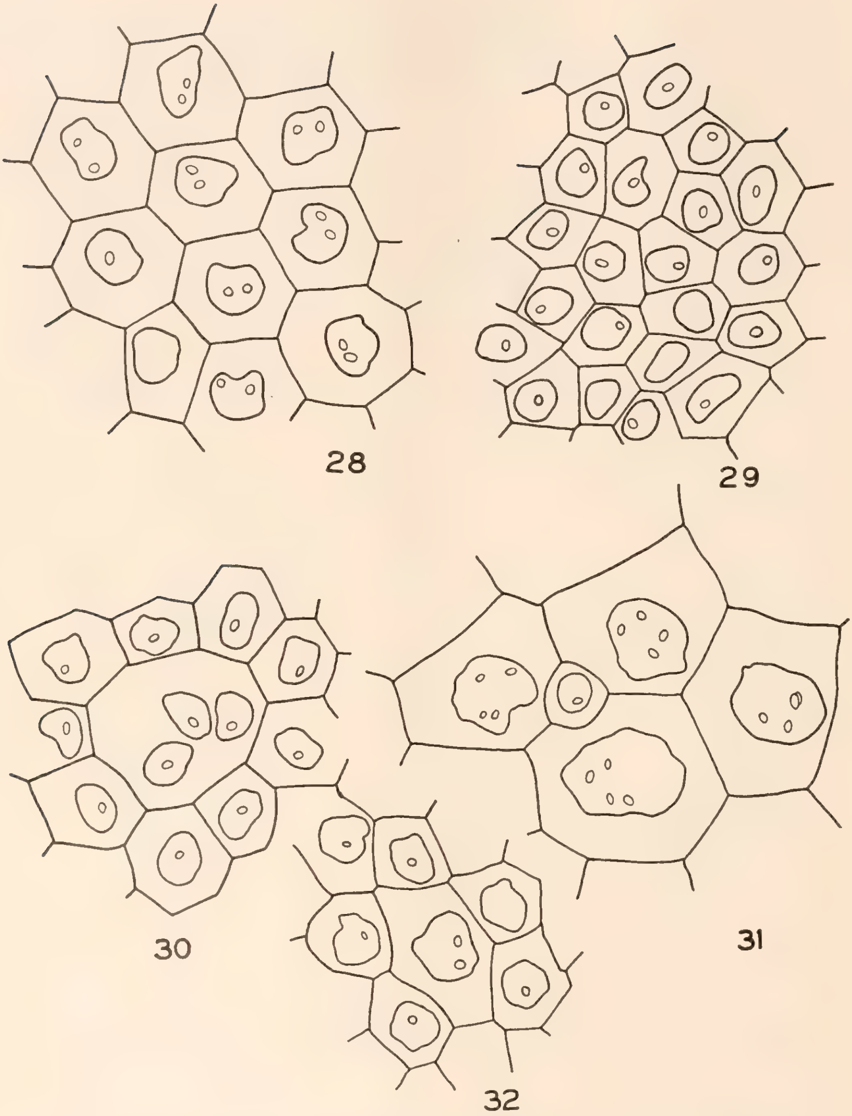
Fig. 24. Triploid metaphase from tail epithelium of 22-day-old triploid animal which developed from an operated egg (Table II). Shows 36 chromosomes (triploid 39).

Fig. 25. Haploid metaphase from 1-day-old androgenetic haploid in early stages of gastrulation.

Fig. 26. Haploid late prophase from tail epithelium of 7-day-old androgenetic haploid.

Fig. 27. Haploid metaphase from cell in tail mesoderm of 10-day-old androgenetic haploid.

Dalcq discovered the same in his preparations and Parmenter located a few diploid divisions in the tissues of some of his older animals which were otherwise predominantly haploid.



Figs. 28–32. Camera lucida drawings of cells and nuclei from tail epithelia. 750 ×.

Fig. 28. From 9-day-old diploid control.

Fig. 29. From 9-day-old androgenetic haploid.

Fig. 30. From 9-day-old androgenetic haploid; shows 3 haploid nuclei in one large cell.

Fig. 31. From 10-day-old androgenetic embryo showing large tetraploid nuclei and cells which predominate epithelium on one side of tail (Table III).

Fig 32. From 10-day-old androgenetic haploid showing diploid nucleus and cell among haploid nuclei and cells.

The nuclear and cell size in haploids has been repeatedly shown to be smaller than in diploids and to this rule these androgenetic frog haploids are no exceptions (Figs. 28 and 29). Observations on haploids and diploids of all ages reveal that this relationship holds whether the observed animals are one day or several days old. It has also been noted that with an increase in chromosome number to triploid and tetraploid there is a corresponding increase in nuclear and cell size (Fig. 31).

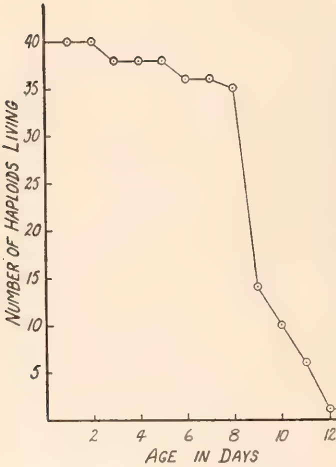
There is a tendency in these haploids for several nuclei (as many as seven have been counted) to occupy a single cell. With this increase in number of nuclei, as with an increase in chromosome number, there is a corresponding increase in cell size (Fig. 30).

The Extent and Uniformity of Development

Studies of groups of androgenetic embryos involving the fixation of representative types at regular intervals do not indicate accurately the extent or uniformity which might be displayed by a total population of such animals. A simple demonstration of these qualities was obtained by allowing each member of a given population of 40 animals to proceed as far as possible in its development. These animals were kept in separate containers under uniform conditions (temperature constant at 20.1° C.). While they were ultimately killed by fixing agents, the same were not applied until the indications were very definite that life would not continue for many hours. The graph presented in Fig. 33 summarizes the data of this experiment. As can readily be seen, up until the fifth day all but two of the original animals were living. From this time until the end of the eighth day there was only a slight change. At this time, however, it was necessary to preserve a large number of them because of their extreme edema. After this pronounced drop the decline is more gradual until the eleventh day after which only one animal remained alive. This one continued to live for several weeks, but, as was expected, it proved to be part haploid and part diploid. The other 39 animals were considered as haploids on the basis of the development which they displayed.

Since these animals were killed by artificial means it seemed advisable to examine some data from earlier groups of androgenetic embryos which had been raised at temperatures averaging 20° C. and in which death was caused by natural agents rather than fixing agents. The data are summarized in Fig. 34 and it is clearly evident that there was a sharp increase in the mortality rate after eight days just as depicted in Fig. 33. Hence the first graph (Fig. 33) can be considered as a correct representation of the survival value of a population of androgenetic *R. pipiens* haploids raised at a temperature of 20° C.

It should not be concluded from these results that androgenetic frog embryos are incapable of further development than that expressed by a 10 or 12-day-old animal raised at 20° C. It is certainly true that the vast majority never go beyond this stage, but the occasional animal will continue longer and while showing abnormalities and a slower rate of growth, it will nevertheless take food and live over several weeks or months. Two animals of the group described in Fig. 34 lived for five weeks and another, which developed from an operated egg in more recent experiments, lived for sixteen weeks. It developed into a sizeable tadpole with small hind limbs. Cytological examination of the tail epidermis has revealed that it was predominantly haploid.



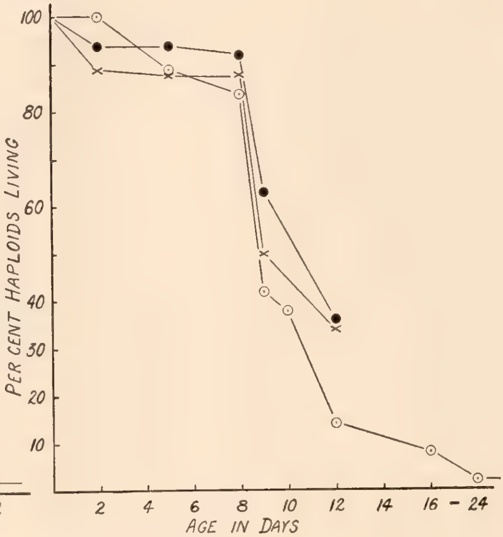


FIG. 33. Graph depicting survival of population of 40 androgenetic haploids which were fixed when it was judged that they could not survive many hours.

FIG. 34. Graph depicting survival of 3 different populations of androgenetic haploids which were allowed to die of natural causes.

The uniformity of a group of androgenetic embryos cannot, unfortunately, be measured by any known unit but must be left entirely to the judgment of the investigator. The fact that the majority of the animals live for eight days, suggests that early development is quite normal and uniform from animal to animal. If, on the other hand, a few animals had died each day and in all stages from cleavage to tadpole, the development could be referred to as un-uniform. The individual animals of the group, the survival of which is described in Fig. 33, were examined every day throughout the duration of the experiment and by means of this examination were compared with one another and with one of the group selected as type. From this study the uniformity can be described as follows: until the end of the third day it was practically perfect, from the third to the fifth day it was fair and from the fifth day on it was rather poor, with differences becoming more pronounced. In other words, as the complexity of structure increased the uniformity of the population decreased.

It is difficult to compare the success (extent and uniformity) of androgenetic development displayed by these *R. pipiens* with the same development of other species. Investigators have used ages rather than stages to describe their results and in using such a unit as days-development, temperature variations become important. Among species of frogs, the androgenetic development described by G. Hertwig (1911) for *R. fusca* and by Dalcq (1932) for *R. esculenta* is no more successful than that reported here for *R. pipiens*. In fact, as far as uniformity is concerned, the results with *R. pipiens* seem to be better. This may be due to the method of operation rather than the species of egg. It has been stated that toad haploids develop better than haploids from the larger frog eggs and that *Triton* haploids develop better than the anurans (P. Hertwig, 1923). A comparative study of amphibian haploidy made at the present time might produce cause to qualify this statement. Until the haploid development of a greater variety of amphibian eggs has been studied it will be impossible to determine whether it is the species of egg, the egg size, the method of initiating haploid development or some combination of these or other factors that makes for greater success in some cases than in others.

Internal Morphology and Development

The typical experimental animals fixed at various ages as recorded in Tables II and III have been sectioned. The description which follows is based on an examination of these sections.

Observations on internal morphogenesis support those on external in showing that development is delayed and abnormal. These facts can be illustrated by an examination of eye development in 3-, 4-, and 5-day-old haploids and controls (Figs. 35–40). In the 3-day-old diploid (Fig. 35) the optic vesicles have extended to the head ectoderm and are in a position to induce lens formation. In the haploid (Fig. 36) the vesicles are smaller, have scarcely reached the head ectoderm and therefore show delayed development. By the end of four days, the control (Fig. 37) shows a well-formed optic cup and lens whereas the haploid (Fig. 38) has advanced only slightly beyond the stage represented by the 3-day-old control and shows only the beginning of lens formation. The 5-day-old haploid (Fig. 40) compared with the control of the same age (Fig. 39) shows an optic cup which is decidedly abnormal. Its dorsal half and the lens are quite similar to the same structures in the 4-day-old control (Fig. 37), but the ventro-lateral lips of the cup fail to grow out leaving a wide choroid fissure. It looks as if the optic stalk in failing to elongate had held in the ventral portion of the cup. Later development does not make up this deficiency in the optic cup, and by a continued proliferation of cells in the retinal layer the structure becomes increasingly abnormal. Only rarely is development more nearly normal. Thus it is observed that while development makes a fairly normal beginning as shown by the vesicle of the 3-day-old, the results as indicated by the 5-day-old and older stages are quite abnormal.

The following survey presents some further outstanding features of haploid internal morphology and morphogenesis as observed from sections of the older stages. They represent observations on the typical haploid.

Nervous System.—An examination of the anterior central nervous system reveals in the oldest haploids a poorly developed brain. In many cases the ventricles are almost entirely obliterated by a marked proliferation of cells or nuclei and a resulting thickening of the brain walls. This condition continues to the posterior end of the medulla. The spinal cord, on the other hand, displays a persisting neurocoele and in the caudal regions is a relatively normal structure. The nuclei are more numerous than in the diploid and in the sections of the older haploids they give way to a vacuolar type of picnosis. The fibre tracts are always indefinite in limitations and have nuclei scattered through them in an abnormal fashion.

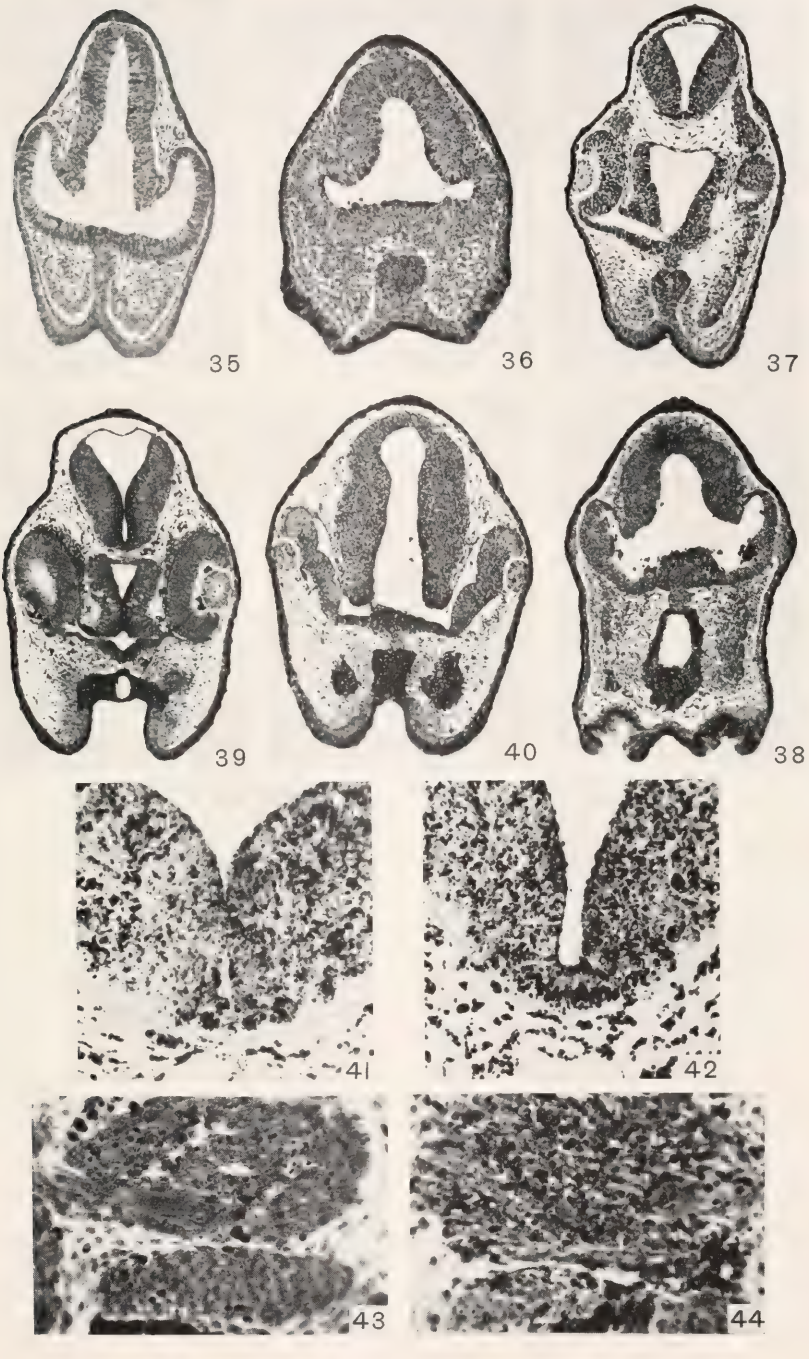
The eye develops abnormally as indicated above. Lenses are absent in many cases and when present are considerably smaller than normal.

FIGS. 35–40. Photomicrographs of sections through optic vesicles and optic cups of haploids and controls, aged 3, 4 and 5 days. 38 ×.

Fig. 35. From 3-day-old control.
Fig. 36. From 3-day-old haploid.
Fig. 37. From 4-day-old control.
Fig. 38. From 4-day-old haploid.
Fig. 39. From 5-day-old control.
Fig. 40. From 5-day-old haploid.

FIGS. 41 AND 42. Sections through the same region of the medulla of 5-day-old control (Fig. 41) and androgenetic (Fig. 42) embryos. Yolk granules are very darkly stained inclusions. 160 ×.

FIGS. 43 AND 44. Sections through the same muscle in the pharyngeal region of 7-day-old control (Fig. 43) and androgenetic (Fig. 44) embryos. 160 ×.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FIGURES 35–40.

The otocyst, unlike the optic cup, differentiates at more nearly the normal rate, but does so abnormally. Instead of one vesicle being at first formed, several develop within the mass of cells which originally arises from the head ectoderm.

The Notochord.—In striking contrast with the nervous system, the notochord is among the best developed and differentiated structures in the androgenetic larvae. By the end of the third day it is well formed and displays a cross-sectional area approximately the same as that of the controls. This same relative size generally persists and when the cells become vacuolated they tend to be smaller and therefore more numerous than in the diploid. Whether or not the more successful differentiation of this structure is related to its early histogenesis is a question of some interest.

The Pronephric Kidney.—This appears slightly later than in the controls and shows fair development. The nephrostomes open into the body cavity and though some difficulty is encountered in tracing the course of the convoluted tubules, they appear to connect with the common duct. This latter is patent and has been traced to an open cloaca in edematous as well as in the more normal androgenetic larvae. This has its interest in that an incomplete lumen in the pronephric duct has been used to explain the edema common to these haploids (Dalcq, 1932). It is evident that such could not be the cause in all cases. The convolutions of the androgenetic kidney are less extensive than in the control kidney of the same age, which suggests a delay in elongation of the tubules. This earlier kidney is vascularized though generally to no avail as the circulation is seldom functional. Evidence for this latter fact often exists in the form of abnormal accumulations of blood cells around the tubules.

Other mesodermal derivatives such as the somites and visceral arches show fair though delayed differentiation. The somites tend to be smaller in cross-sectional area and to be underdeveloped in the thin dorsal extensions lateral to the nerve cord. The muscle cells are smaller and less compactly grouped.

The Circulatory System.—The circulatory system is functional in very few cases though the heart beats in many. The differentiation of the heart is considerably delayed and is generally about 24 hours behind the control in showing its first pulsations. The larger vessels can be located and traced, but the development of capillary connectives is doubtful. This latter failure is suggested by the patches of blood cells which accumulate in various regions of the body not normally associated with blood formation. Only in the occasional haploid can a good capillary circulation be located in any part of the body. The blood cells are generally less numerous, are smaller, and contain more yolk granules. They often contain 2 or 3 nuclei after the yolk platelets have disappeared.

The Gut.—The gut is markedly retarded in its differentiation. This is most emphatically shown by the fact that in a 9-day-old edematous haploid the gut appears as an almost straight tube whereas in the control of the same age it is considerably coiled. The walls of this short gut are thicker and the cells are packed with yolk. The derivatives of the gut likewise differentiate rather tardily. For example, the lungs, arising from the fore-gut, are in about the same stage of development on the ninth day as they were on the seventh day in the controls. This 2-day delay in differentiation is, however, not common to the whole animal.

The Ectoblast.—The ectoblast in its differentiation more closely parallels the controls than any other part of the embryo. Oral suckers, olfactory pits, mouth parts and operculum all differentiate quite normally and at approximately the normal time. The ectoderm, at first wrinkled and thicker than in the controls, becomes thinner as the animal becomes edematous. Tumor-like proliferations of the ectoderm occasionally appear, and are not unlike those shown by frog embryos treated with weak solutions of 2,4-dinitrophenol (Dawson, 1938), or with high temperatures (Hoadley, 1937), or developed from over-ripe eggs (Witschi, 1930).

Yolk Supply

Only a brief examination of the sections of these haploids was necessary to show that yolk disappears more slowly from the cells of the haploid than from the diploid. Since it was felt that considerable importance could be attached to this observation studies of yolk content were made along with studies of morphology. These are considered but the beginning of future studies which may throw some light on the causes of haploid deformities.

Until the haploids and controls are 4 days old (20° C.) the yolk content of the cells in all regions of the embryo is so great that microscopic comparisons are without value. In animals varying from 4 to 7 days a comparative examination of the same organs in haploids and diploids of the same age reveals a greater quantity of yolk in the cells of the haploid (Figs. 41 to 44). Within these age limits this difference holds for all tissues of the embryos though it is more apparent in some than in others. In haploids older than 7 days the yolk supply of some tissues (ectoderm of 8- and 9-day-olds) is completely exhausted while in others (the gut) it is still possible to observe a greater quantity in the cells of the haploid. It can be noted further that differentiation seems to be more delayed in regions most richly supplied with yolk. Further observations, and if possible measurements of yolk content, are necessary before it can be stated that the cells of a haploid tissue do not approach a normal stage of differentiation until their yolk supply has been reduced to the normal extent. It is hoped that future experiments may clear up this matter and provide a basis for definite conclusions.

DISCUSSION

The results of these experiments indicate that the operation by which the maturation spindle is removed from the egg is successful. Approximately 90 per cent of the operated eggs develop as haploids, the haploid nucleus being that of the sperm. It has been shown that under the influence of this nucleus development proceeds for 8–10 days (20° C.) and produces a tadpole showing considerable differentiation. This is abnormal, however, and only future experiments on other eggs will indicate whether more normal haploid development is possible among the Salientia. The uniformity displayed by these populations of haploid embryos has been described as good over the first 3 days and fair from the third to the fifth days. This degree of uniformity appears to be a distinctive feature of this material for it is not clear that similar results have been previously obtained with other eggs and methods.

It is shown, therefore, that haploid embryos of suitable quality are made available in sufficient numbers for physiological studies and measurements. The abnormalities which they demonstrate occur in sufficient uniformity to make the study of their cause attractive and possibly productive. And, from another angle, they become particularly valuable as a material for hybridization experiments involving the mixing of the cytoplasm of one species with the nucleus of another.

The subject of special interest in connection with this report is the abnormal retarded development and reduced viability of these haploids, which, it is clear, must be related to the presence of only the haploid chromosome complement. Recessive genes, lethal or otherwise, unsuppressed by dominant allels would, if present in the sperm nucleus, find definite expression in these haploids. It seems hardly probable, however, that these would occur with such regularity within the male chromosomes as to produce, for example, a similar reduction in the length of the neural plate in almost every haploid in a population of 40 experimental animals. It is more logical to associate such a departure from the normal with the presence of a haploid nucleus within a quantity of cytoplasm normally associated with a diploid nucleus.

Several hypotheses have been proposed to account for these haploid abnormalities and Fankhauser (1937) finds in them a common idea: a disturbance of the metabolism of the haploid cells. As to the nature of this disturbance there is no clear understanding, but it is presumably due to a supply of yolk and cytoplasm excessive for the haploid nucleus. There is some evidence in support of this hypothesis in the results of these investigations. It has been noted that the yolk supply disappears more slowly from the cells of the haploid than from the diploid. It has also been noted that differentiation is delayed and abnormal and that the delay appears to be more pronounced in tissues containing the greatest amount of yolk. From these observations it is not unreasonable to link excess yolk with delayed and abnormal differentiation. Additional supporting evidence comes from experiments on merogonic development. In the production of merogonic haploids the quantity of cytoplasm is more or less reduced and the normal karyoplasmic ratio tends to be restored. One such fragment of a *Triton taeneatus* egg developed through metamorphosis and constitutes the most successful case of amphibian haploidy on record (Baltzer, 1922; Fankhauser, 1938). Thus a decrease in egg cytoplasm to conform with the haploid nucleus may have permitted more normal development.

While these observations suggest a cytoplasmic influence as being responsible for the abnormalities, there is evidence which indicates that the influence in some cases arises from the nucleus. For instance, investigations of the early cleavage stages of merogonic egg fragments of *Triton palmatus* and *Triturus viridescens* have shown an unequal distribution of chromosomes (Fankhauser, 1932*c* and 1934*c*). This has been held responsible for the high mortality rate which it is customary for these merogonic embryos to show before or during gastrulation. The same explanation has been extended to the non-viable blastulae and gastrulae among *Triturus viridescens* embryos (Fankhauser and Kaylor, 1935). It is impossible, however, for any such alteration in nuclear structure to be responsible for the abnormalities of the typical frog haploid since a complete haploid complement of chromosomes has been observed in all cases studied.

Only when these studies have been extended and more is known concerning nucleo-cytoplasmic reactions whereby differentiation is brought about will it be possible to state with any certainty the conditions within the cytoplasm or nuclei of these haploids which make more normal development impossible.

SUMMARY

1. A technique is described by which the second maturation spindle and so all of the maternal chromatin can be removed from the egg of *R. pipiens* following its activation and penetration by the sperm.

2. The operation as applied to this egg is considered satisfactory for a large number can be treated in a short time, the maturation spindle is removed with certainty, and the slight amount of cytoplasm removed has no destructive effect on the development which follows.

3. Between 90 per cent and 100 per cent (generally 100 per cent) of the operated eggs undergo first cleavage simultaneously with the controls, and of these the majority develop for eight days, a few considerably longer.

4. The development of androgenetic haploids compared with diploid controls of the same age is abnormal and delayed. Certain features of external and internal morphogenesis are described.

5. It is shown that 90 per cent of the operated eggs can be expected to develop as haploids. This haploid nuclear condition remains unchanged until the final stages of development and then is altered only by the presence of a very few diploid nuclei.

6. As is typical for haploids, the cells and nuclei of these androgenetic embryos tend to be smaller than those of the diploid controls.

7. The development shows a high degree of uniformity from animal to animal over the first five days after which the differences become more pronounced.

8. Yolk disappearance from the cells of the haploids is notably delayed.

9. The ease of production and the success of development of *R. pipiens* androgenetic embryos seems to provide one of the best possibilities so far encountered for the study of haploid morphogenesis from eggs which normally develop as diploids.

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THE ILLUMINATION OF THE EYE NECESSARY FOR DIFFERENT MELANOPHORIC RESPONSES OF FUNDULUS HETEROCLITUS[1]

EARL O. BUTCHER

(From the Biological Laboratory, Hamilton College, and the Mount Desert Island Biological Laboratory, Salisbury Cove, Maine)

It has been established by previous investigations (Butcher, 1938) that the upper region of the retina of *Fundulus* is related to the paling of the body and the lower region to the darkening of the fish. During the course of these investigations many problems were encountered and left unsolved. Among them were: (1) Why does illumination of the lower region of the retina with a Mazda lamp cause most fishes to darken, but illumination of the upper region with a Mazda lamp induce only a few to pale? (2) How much of the regions have to be illuminated to elicit the related melanophoric responses? (3) To what extent does illumination of the upper region have to be eliminated in order that darkening can be induced by illuminating the lower region? (4) Is the paling response more easily elicited when light is entirely eliminated from the lower region?

The cause of a fish assuming a paler shade in a shaded white box than in a brightly lighted gray box was also investigated. It seemed that the assumption of the shade of the background by the fish might depend upon the ratio of the direct light coming from above and the reflected light from below which enters the eye as Sumner (1911), and Sumner and Keys (1929) have contended to be the case for the flounder.

The present investigations show that when a Mazda lamp is placed above fishes in a black dish, the image of the lamp falls upon enough retinal receptors in the lower region of the eye to induce darkening of the body, but when the same lamp is placed below fishes and its image falls upon the retinal receptors of the upper region, this image is not large enough to induce the paling response. Illumination of a large area of the upper region is, therefore, necessary to induce the paling response. Paling is also more easily elicited when only the upper region is illuminated. Illumination of the lower region induces darkening only when there is very little illumination to the upper region.

The melanophore response elicited by illuminating the lower region of the eye may be reduced by the simultaneous illumination of the upper region. For instance, illumination from above to the lower region of the eye causes darkening of the body when there is little reflected light from the bottom of a black dish to the upper region. If a gray bottom is used, a greater percentage of the light is reflected to the upper region of the eye, a greater inhibitory reaction is induced, and the degree of darkening of the body is reduced. If this gray background is more intensely illuminated from above, then the reflected light is greater to the upper region. The same ratio, however, persists between the direct and the reflected light, a proportional inhibitory effect is induced and the fish assumes the same shade as when the intensity of the direct light is lower.

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[1] Reported before the American Society of Zoologists at the December, 1938, meetings. *Anat. Rec.*, vol. 72 (suppl. no. 4), p. 80.

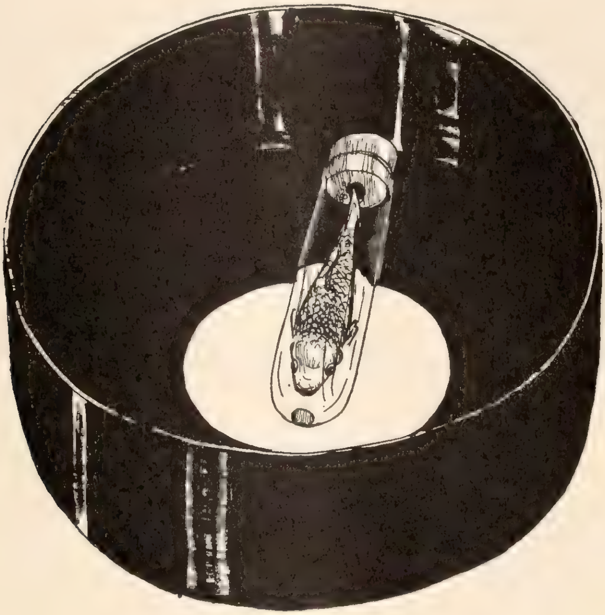

FIG. 1.

The Conditions Affecting the Paling Response

The sides of crystallizing dishes, 20 cm. in diameter, were lined with black paper which reflected approximately 1 per cent of the light striking it. Fishes were placed in these dishes containing water 4 cm. deep, and the top of the dish was covered with black paper. When the fishes were illuminated through the glass bottom by a 60-watt, inside-frosted Mazda lamp, placed 18 cm. below the dish, a few of them became slightly pale and the rest assumed an intermediate shade. The fishes, in this instance, were receiving about 200 footcandles of illumination (determined by a Weston photronic illuminometer) from the lamp while the brightness of the lamp was approximately 58,000 footlamberts.[2]

If a piece of white paper or opal glass, as large as the bottom of the dish, were inserted between the source of illumination from the Mazda lamp and the bottom of the dish, the fishes readily paled even when the brightness below them was 1 footlambert or less. The image of the white bottom being larger than the image of the bulb alone fell on a great many more retinal receptors. Paling, therefore, depends mainly upon the size of the white area seen by the fishes.

TABLE I

Relation between body size and diameter of circle below fish necessary to induce paling.

Number examined	Length of body	Width of body at level of eyes	Diameter of circles and number paling			
			9 cm.	7 cm.	5 cm.	3 cm.
	mm.	*mm.*				
6	40–45	5–6		6	6	2
59	50–60	7–8	59	48	16	0
15	60–70	9–10	15	14	7	0
7	70–80	10	7	7	0	0

As a means of determining the size of the white area necessary for inducing paling of the body, fishes were enclosed in glass tubes (16 mm. inside diameter) which had small openings at both ends for the circulation of water. These tubes with the fish inside were placed over circles of white paper in such a way that the fish's head was above the center of the circle (Fig. 1). The circles were then either illuminated from above or from below.

To induce paling of fishes 50 mm. in length, circles 7 cm. in diameter were usually necessary (Table I). When the fishes were over 3 cm. circles, an intermediate shade was always assumed. Fishes paled equally as well when the circles were exposed to 4.5 footcandles as to 450 footcandles from above.

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[2] The author is greatly indebted to Mr. Frank Benford of the General Electric Company, Schenectady, N. Y. for determining the brightnesses with a Luckiesh-Taylor Brightness-Meter.

Figure 2, which is drawn to scale, shows approximately the size of the image in the upper region of the eye when the fish was over the various circles. It is evident that a large area of retinal receptors must be stimulated before paling is induced. Images of 5 and 7 cm. circles, being nearly the same size (Fig. 2), caused only slight differences in the degree of paling. It might have been better to use a square tube to hold the fish, since a round tube probably acted as a cylindrical lens, and the fish did not get an image quite like the white circle and even illumination.

Fishes of various lengths were tested in tubes of the same size over circles of white paper. Since the body of a small fish covered less of the 3 cm. circle than did the body of a large fish, the small fish saw more of the circle (Figs. 3 and 4), and assumed a paler shade over the 3 cm. circle than the large fish (Table I).

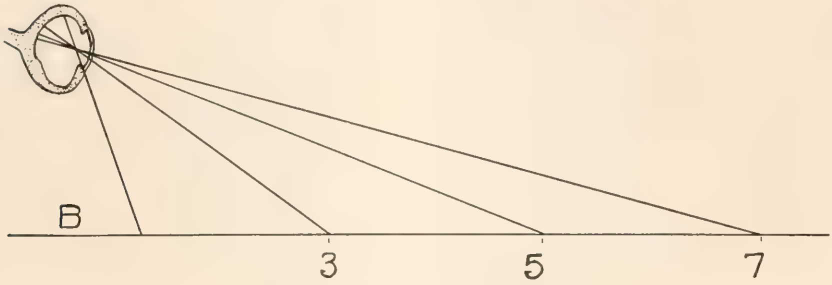
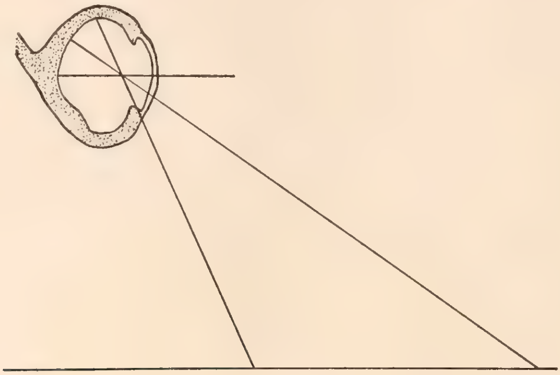


FIG. 2. Sizes of images in upper region of eye when fish was over circles with various diameters. Dorsal ventral diameter of eye—4 mm.; width of head—8 mm.; distance between eye and background—6 mm.; *B.*, background; 3, 5, 7, boundaries of 3-, 5-, and 7-cm. circles.

Paling, therefore, depends greatly upon the size of the white area below the fish.

In previous experiments (Butcher, 1938) where blinders were used in covering the eyes, there was some evidence that illumination from above tended to inhibit the paling response or that paling was more easily elicited when the lower region of the eye was not simultaneously illuminated. There is no way of confirming this observation with a white background below and illumination from above because variation in the illumination from above causes a proportional variation in the reflected light from below. Likewise, if a white bottom is illuminated from below, causing the fish to pale, then illumination cannot be added from above in any way so that paling will not persist.

Whether or not light from above was inhibitory to the paling response was investigated in the following way. A circle of white paper which would induce paling when placed below a fish and illuminated from above (Fig. 1) was cut into halves. The fish in the glass tube was then placed over half of this circle in such a way that the axis of the fish corresponded with the diameter of the circle (Fig. 5). Fishes arranged in this manner failed to pale, because illumination of the lower regions of both eyes was enough to inhibit any response elicited by the reflected light to the upper region of one eye. Even if the diameter of the circle was greatly increased, paling was not induced in most instances. When the eye which was not over the white semicircle was enucleated, the fish immediately paled, for now the inhibition



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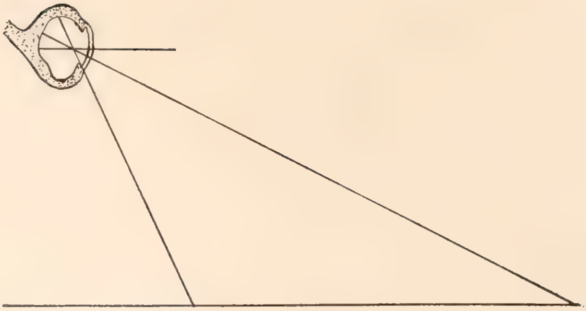

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Figs. 3 and 4. These figures illustrate that more of the 3 cm. circle is seen by the small fish than by the large fish. The image in the small fish covers approximately 15 per cent more of the upper region of the retina.

Fig. 3. Eye of fish 80 mm. long, and 10 mm. wide at eye level.

Fig. 4. Eye of fish 40 mm. long, and 6 mm. wide at eye level.

resulting from illuminating the lower region of one eye was not enough to prevent the influence of the upper region of one eye. These experiments definitely showed that the paling response was more easily elicited when the lower regions of the eyes were not so intensely illuminated.

The Conditions Necessary for Inducing the Darkening Response

To determine the intensity of illumination of the eye necessary for inducing complete darkening of the body, fishes were placed in glass dishes lined with black paper. These dishes were about 20 cm. in diameter, 8 cm. deep, and contained water 4 cm. deep. A cylinder lined with a light-proof, black paper enclosed the dishes. The top of the cylinder was covered with opal glass, and a Mazda lamp, inside-frosted, was suspended above the cylinder as the source of illumination. The diffusing opal glass was 18 cm. from the surface of the water in the dish. Two small openings were made in the side of the cylinder. One was used for observing the fish and the other was large enough for transferring fish in and out of the black dish. The temperature of the water was kept at about 16° C. It was always ascertained if fish would assume both pale and dark shades before they were used for the experiments.

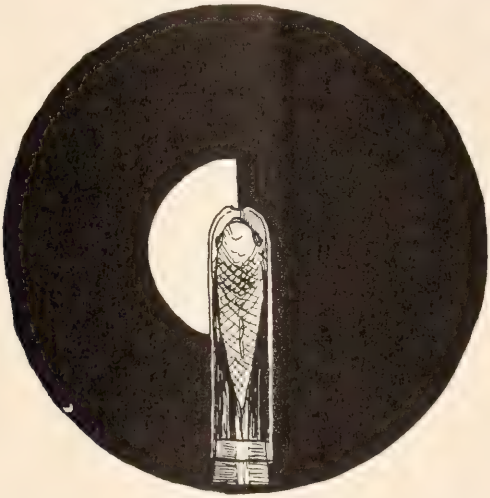

FIG. 5. This figure shows how a fish was placed over a semi-circle so that the upper region of only one eye was illuminated by reflected light from below.

In investigating the effect of illuminating the lower region of the retina with different intensities three or four fishes were placed in the dish and allowed to remain for 20 minutes. Meanwhile, control fishes in other black dishes outside of the cylinder were being exposed to intensities from Mazda lamps which definitely induced maximum darkness. At the termination of 20 minutes, one studied the experimental fishes through the small hole in the side of the cylinder and observed their shades. In order to determine more definitely how many were completely dark and the correctness of the observations made in the experimental dish, the observer then viewed the control fishes, and without changing his field of vision, he quickly transferred an experimental animal into the control dish. This method involved only a few seconds and reduced the possibilities of error as much as any method used. Fishes were tested only once and then discarded.

When the intensity of illumination reaching the fishes was reduced to 2 footcandles, the majority of them failed to completely darken. Exposing 60 fishes to this intensity, 40 per cent of them became completely dark, 20 per cent darkish, and 40 per cent intermediate. The tables in the article by Brown (1936) show that an intensity of illumination of 1.75 footcandles caused complete dispersion of melanin in many *Ericymba buccata* Cope, the silver-mouthed minnow. Danielson (1938) reports that complete melanophore change appeared to occur at and above 1 footcandle in *Nocomis biguttatus* Kirtland. There are undoubtedly variations between different species and variations in threshold between different individuals. When the intensity was increased to 3.5 footcandles and 54 fish were tested, 45 became completely dark, and the other 9 had a darkish appearance.

Since it was necessary to stimulate a large area of receptors in the upper region of the retina in order to induce paling of the body, a few investigations were undertaken to determine the size of the source of light to the lower region necessary to cause the darkening response. In place of the opal glass covering of the cylinder, a black lid was substituted. This covering contained a central aperture, the size of which could be varied. When this aperture was 1.5 cm. in diameter and the fish were receiving 2 footcandles, about the same percentage (40 per cent) became maximally dark as when they received 2 footcandles through opal glass. Only 25 per cent, however, definitely assumed a maximum darkness when the diameter of the aperture was reduced to 1 cm. and they received an intensity of 2 footcandles.

Some *Funduli* thus become maximally dark when receiving an intensity of 2 footcandles from a source of light 1 cm. in diameter. The diameter of the image formed by a source of light 1 cm. in diameter and 18 cm. from the eye is only about .085 mm. or 85 micra. Whether or not more than 25 per cent of the fish will be induced to darken when receiving an intensity greater than 2 footcandles from a source 1 cm.

in diameter has not been determined. At least, a much smaller image induces darkening than the image necessary to elicit paling of the body. There are also undoubtedly individual differences in threshold.

Evidence that the Shade of the Fish Depends upon the Ratio between the Light from Above and the Light from Below Entering the Eye

Observations made by Sumner (1911), and Mast (1916) show that the shade of the flounder's body does not depend upon a visual comparison between its body surface and the background. It seems more probable from their experiments and those of Sumner and Keys (1929) that the ratio between the light coming from above and that reflected from below supplies the stimulus to the eye which enables the fish to assume a certain shade.

To learn if the ratio of light was responsible for the shade assumed by *Fundulus* it was first necessary to secure backgrounds which ranged in shade from white to black, to determine the response of the fish with each background, and the ratio of the direct to the reflected light in each instance. Various gray papers were used for these backgrounds and these were placed in the bottoms of large crystallizing dishes, the sides of which were lined with black paper. These crystallizing dishes were held with clamps about two feet from a table in a dark room. For illuminating the bottom of the dish, Mazda inside-frosted lamps were placed both above and below the dishes.

The response of the fish, the kind of bottom, and the brightness of the bottom when illuminated only from above are recorded in Table II. The responses of 15 fish were usually determined in each of these experiments. Gray 1, gray 2, and gray 3 were very close to neutral 6, 5, and 3, respectively, of the Munsell "Book of Color." [3]

The higher intensities induced about half of the fish in the dish with gray bottom 2 to pale. Reducing the brightness of the bottom to .1 footlambert when the intensity from above was .5 footcandle caused only a few to pale (Table II). Apparently the percentage of reflected light has to be greater than it is from bottom 2 (50 : 10) to induce all to pale.

If the fish assumes the shade of the background below because of the ratio of the light from above to the light from below entering the eye, then fish should pale over gray 3 when its brightness is increased by illumination from below, and a ratio is established which is known to induce paling of the body. To test this hypothesis the Mazda lamp, inside-frosted, was turned on under gray 3 while the intensity of illumination from above remained 5 footcandles. When the brightness of this paper was 3 footlamberts or a ratio (5 : 3) existed which induced paling over a white background, the fish, likewise, paled over this gray 3. With added illumination (400 footcandles) from above so that the ratio was 50 : 3.3, an intermediate shade was quickly assumed.

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[3] "The atlas of the Munsell Color System," Munsell Color Company, Inc., Baltimore, Maryland.

If either gray 1, 2, or 3 were illuminated only from below and their brightness was 1 footlambert (ratio in this instance is 0 : 1), all fish quickly paled. With no illumination from above fish could undoubtedly be induced to pale when the brightness of the bottom was much less than 1 footlambert for they have paled when the background was .2 footlambert in brightness and the intensity from above was .5 footcandle (Table II).

TABLE II

Light relations and shade of fish's body with different shades of paper below fish. *P.*, pale; *SP.*, slightly pale; *Int.*, intermediate.

Shade of paper below fish (sides of container black)	Intensity of light from above footcandles	Brightness of background below fish footlamberts	Ratio of light from above to brightness of background below	Shade assumed by fish
White	400	220	50 : 27	P
	10	5.5	50 : 27	P
	5	2.75	50 : 27	P
	.5	.275	50 : 27	P
Gray 1	400	160	50 : 20	P
	10	4	50 : 20	P
	5	2	50 : 20	P
	.5	.2	50 : 20	P
Gray 2	400	80	50 : 10	50% P, 50% SP
	10	2	50 : 10	50% P, 50% SP
	5	1	50 : 10	50% P, 50% SP
	.5	.1	50 : 10	20% P, 80% SP
Gray 3	400	24	50 : 3	Int.
	10	.6	50 : 3	Int.
	5	.3	50 : 3	Int.
	.5	.03	50 : 3	Int.

Some fish, therefore, paled when the ratio of direct to reflected light was 50 : 10. Fish failed to pale over a gray background below when lighted from above because this background did not reflect enough light in comparison to the light coming from above. If the gray background were illuminated from below so that its brightness was increased, then the fish paled.

SUMMARY

Only a small area of the lower region of the eye of *Fundulus* needs be illuminated to induce a darkening of the body, since as little light as 2 footcandles coming from a source 1 cm. in diameter and 18 cm. above the fish elicits the darkening response. Darkening cannot be induced by illuminating the lower region when there is much illumination to the upper region of the eye.

For eliciting paling of the body, a large area of the upper region of the eye must be illuminated. This is shown by experiments with fish over circles. Regardless of the brightness of a circle 3 cm. in diameter beneath the fish, those 50 mm. in length failed to pale. When the illuminated circle was increased in size, fish paled readily. Paling, therefore, depends greatly upon the size of the white area seen by the fish. A Mazda lamp arranged below a fish so as to illuminate the upper region of the eye thus fails to induce paling because its image does not fall upon enough retinal receptors. Paling is more easily elicited when the lower region of the eye is not illuminated at the same time that the upper region is illuminated.

The ratio between the direct and the reflected light, known to exist with a white background below the fish, has been created with gray bottoms by illuminating them both from above and below. Gray backgrounds illuminated in this way have caused fish to pale readily. The shade assumed by *Funduli*, therefore, depends upon the ratio between the direct and the reflected light entering the eye.

As the percentage of reflected light to the upper region of the eye is increased, there is induced a proportional increase in the inhibitory reaction which causes a reduction in the degree of darkening of the fish.

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OBSERVATIONS UPON AMPHIBIAN DEUTOPLASM AND ITS RELATION TO EMBRYONIC AND EARLY LARVAL DEVELOPMENT[1]

ARTHUR N. BRAGG

(*From the Zoölogical Laboratory of the University of Oklahoma*)

During early ontogeny, several distinct morphogenic processes proceed more or less synchronously whereas others tend to alternate (Richards, 1935). In the exponential period, described by Schmalhausen (1930), mitotic activity dominates; but with the onset of gastrulation, the mitotic rate falls in close correlation with an increase in differentiation (initiation of the parabolic period). It is at this time, just as the primary caudo-cephalic axis is about to be laid down, that the first embryonic organizers become evident in the dorsal blastoporal lip (at least in Amphibia) and also that important mitotic centers are set up which feed cells into specific regions where they later differentiate into various anlagen, in some cases, at least, under the influence of induction (Derrick, 1937; Self, 1937; Bragg, 1938; Jones, 1939). Behind these more or less morphological manifestations are the actions of the genes, inductors, possibly hormones, etc. which, working through the visible morphological configurations of the cells or their parts, actually are the basic underlying factors in the production of the embryo, and hence of the adult body.

From these considerations, it is evident that the basic factors in embryonic development are essentially physiological, rather than morphological, in character. Studies of cell-migrations or of morphogenic movements (Vogt, 1929; Wetzel, 1929; Gräper, 1929; Pasteels, 1936; etc.), or mitotic indices (Minot, 1908; Self, 1937; Derrick, 1937; Bragg, 1938; Jones, 1939, etc.), and all similar attacks upon the problem of embryological organization cannot, each method of itself, explain morphogenesis. Such studies are valuable mostly as indicating changes in the morphological configurations of parts which in turn are indirect evidences of the basic physico-chemical changes in the protoplasm, a detailed understanding of which can only be attained by physiological methods. Sometime in the future, therefore, we may expect a synthesis of the observations made by the various methods now in use wherein the relationship between cell division, and mitotic centers, induction, cell-migration, increase in size, histological differentiation, problems of cell-size and body-size, nucleo-cytoplasmic ratios, the mode of genic action, etc. will all be correlated into one basic biological principle, only fragmentary glimpses of which any one of us now sees by the results of his own special method.

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[1] Contribution from the Zoölogical Laboratory of the University of Oklahoma, No. 199.

It is well established that the yolky materials in an egg of an animal constitute reserve food which is utilized during some phase of ontogeny as a source of energy, of building materials, or of both: but at just what phase of development and for what processes they are utilized by the animal has apparently received but slight attention (see, however, Saint-Halaire, 1914). During a recent study of the relation of cell division to early embryonic organization of a toad (Bragg, 1938), it was observed that the yolk granules maintained their initial sizes, shapes, and appearances at least to the stage in which the neural tube closed. From indirect evidence, it was also strongly suspected that the embryo did not increase materially in protoplasmic mass up to this stage of development. If these two conclusions were substantially correct, this could only mean that the yolk was not used during the exponential period nor even during the earlier portion of the parabolic period wherein all of the anlagen of the major organ systems were laid down. In other words, the yolk contributed neither energy for the very actively katabolic process of cleavage nor materials for the increase in the size of the body up to this stage of development in the embryos of the species investigated.

Since these observations were somewhat incidental to the main subject of the former paper, and, further, since the yolk must bear important relationships to some of the ontogenetic processes indicated above, it seemed wise to study the yolk in greater detail in order to establish when and where its utilization begins and, so far as possible by the methods used, for what embryological processes it is utilized. It is also of interest to ascertain whether the species used in the former study (*Bufo cognatus*) is peculiar in these matters or whether other amphibian species manifest the same phenomena.

MATERIALS AND METHODS

The embryos used were those of *Bufo cognatus* Say, *B. woodhousii woodhousii* (Girard), *Rana sphenocephala* (Cope), and *Scaphiopus hammondii* Baird, all from the vicinity of Norman, Oklahoma.[2] Prepared slides of early cleavage, of the blastula, and of the gastrula of the California newt, *Triturus torosus*, were studied also for comparison with the anuran embryos.

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[2] I am indebted to the following for the use of slides of embryos and larvae prepared and owned by them: to Mr. Virgil Johnson for all stages of *B. w. woodhousii;* to Dr. Minnie S. Trowbridge for embryos of *Scaphiopus;* and to Mr. Robert Taylor for larvae of *Scaphiopus.*

The species of *Scaphiopus* used is the same as that called tentatively *S. bombifrons* Cope by Trowbridge and Trowbridge (1937). In a forthcoming paper, Dr.

The methods were those commonly employed for embryological work. Embryos and larvae were fixed in one of several different fixing fluids (Smith's, Goldsmith's, and Bouin's, most commonly), dehydrated with ethyl alcohol, embedded by the method of Hamlett, and serially sectioned (6–12 micra). Heidenhain's haematoxylin, alum haematoxylin, and alum cochineal were the principal stains used. The exact procedure made little difference for the purpose of the study. Observations upon living embryos and larvae of all species used except the newt were also made.

Following the same method as earlier (Bragg, 1938), the yolk granules in selected regions were drawn under oil-immersion lenses by means of a camera lucida, all carefully to the same scale. The pictures so obtained were then compared with each other and with the details of structure as seen in the microscopic fields. The facts

Minnie S. Trowbridge and the author will show that the species name, *bombifrons* is not a synonym for *hammondii* as assumed by Wright and Wright (1933) and that the species in question here is *S. hammondii*.

EXPLANATION OF FIGURES

All figures in the plates drawn by camera lucida and to the same scale in order that they may be compared with one another directly. All are of complexes of yolk granules characteristic of the region given for each except Figs. 44 to 49.

A. Blastula No. 12A1, comparable to Bragg, 1938, Stage *A*.
FIG. 1. Micromere.
FIG. 2. Intermediate zone.
FIG. 3. Macromere.
B. Gastrula No. 263A2, comparable to Stage *C* (Bragg, 1938).
FIG. 4. Dorsal ectoderm.
FIG. 5. Just inside the dorsal lip of the blastopore.
FIG. 6. Anterior ectoderm (opposite the yolk plug).
FIG. 7. Dorsal blastopore region. The blastoporal groove between a cell of the dorsal lip (left) and a cell of the yolk plug.
FIG. 8. Condition a short distance inside the blastopore at the dorsal lip. Condition of yolk intermediate between those shown in Figs. 5 and 10.
FIG. 9. Ventral lip of the blastopore.
FIG. 10. Inner yolk mass.
C. Stage of the crescentic blastopore, No. 54A4, comparable to Stage *B* (Bragg, 1938).
FIG. 11. Micromere near the animal pole.
FIG. 12. Innermost yolk cells.
FIG. 13. Region of the dorsal blastoporal groove; compare with Fig. 7.
D. Neural plate stage. No. 75XA1, comparable to Stage *D* (Bragg, 1938).
FIG. 14. Neural plate.
FIG. 15. Dorsal endoderm.
FIG. 16. Lateral mesoderm.
FIG. 17. Lateral ectoderm.
FIG. 18. Ventral ectoderm.
FIG. 19. Ventral yolk mass.

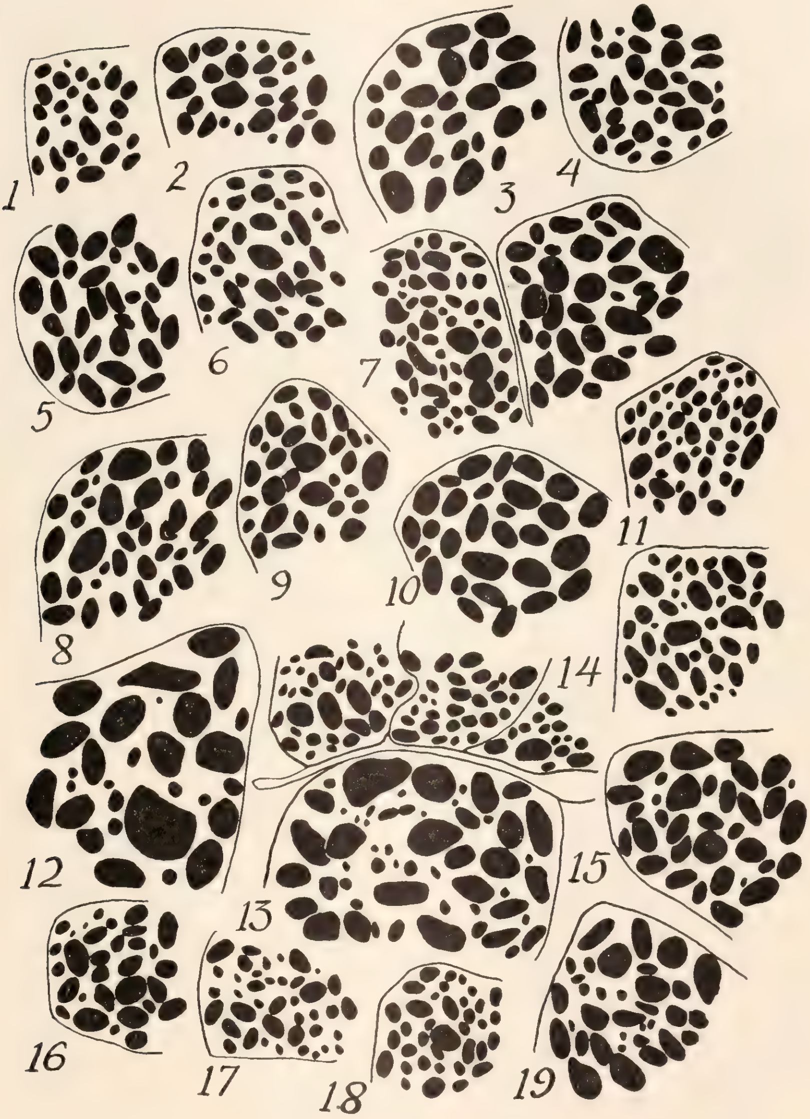
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Plate I. *Bufo cognatus*

gathered in this manner were then correlated with the known stage of development of the individual animals from which the slides had originally been made.

Observations

The distribution and sizes of the yolk granules of all species used followed the general pattern already described for *Bufo cognatus* (Bragg, 1938). Briefly, a gradient of size exists, the smallest granules being mostly located in the animal region, the largest in the vegetal portion of the egg. Species differ in the absolute sizes of the granules but the mode of distribution is the same in all. During cleavage, three types of blastomeres become recognizable, each easily differentiated from the others by the type of yolk granules contained.

Explanation of Figures—Plate II

Fig. 20. Dorsal mesoderm.
Fig. 21. Notochord.

E. Neural tube not quite closed. No. 112A3, comparable to Stage *E* (Bragg, 1938).

Fig. 22. Dorsal mesoderm.
Fig. 23. Superficial lateral ectoderm.
Fig. 24. Neural tube.
Fig. 25. Lateral endoderm.
Fig. 26. Endo-chordo-mesoderm.
Fig. 27. Lateral mesoderm.
Fig. 28. Dorsal endoderm.
Fig. 29. Notochord.

F. Neurula. No. 273a.

Fig. 30. Optical vesicle.
Fig. 31. Ectoderm adjacent to the adhesive organ.
Fig. 32. Brain.
Fig. 33. Adhesive organ.
Fig. 34. Ventral ectoderm.
Fig. 35. Notochord.
Fig. 36. Mesenchyme of the head.
Fig. 37. Lateral mesoderm.
Fig. 38. Ventral yolk mass.
Fig. 39. Lateral ectoderm.
Fig. 40. Dorsal endoderm.
Fig. 41. Somite mesoderm.
Fig. 42. Ectoderm of the head.
Fig. 43. Nerve cord.

G. Larva of 3 mm. total length. No. 134 2-2.

Fig. 44. Ventral yolk mass.
Fig. 45. Mesoderm. Note that the yolk is being used.
Fig. 46. Superficial ectoderm. Yolk nearly gone.

H. Larva of 5 mm. total length. No.141E1.

Fig. 47. Myomere of the tail.

Fig. 48. Section of the nerve cord dorsal to the yolk region (Fig. 44). Yolk granules scattered and small.

Fig. 49. Outline of a fold of superficial ectoderm with only the yolk granules shown.

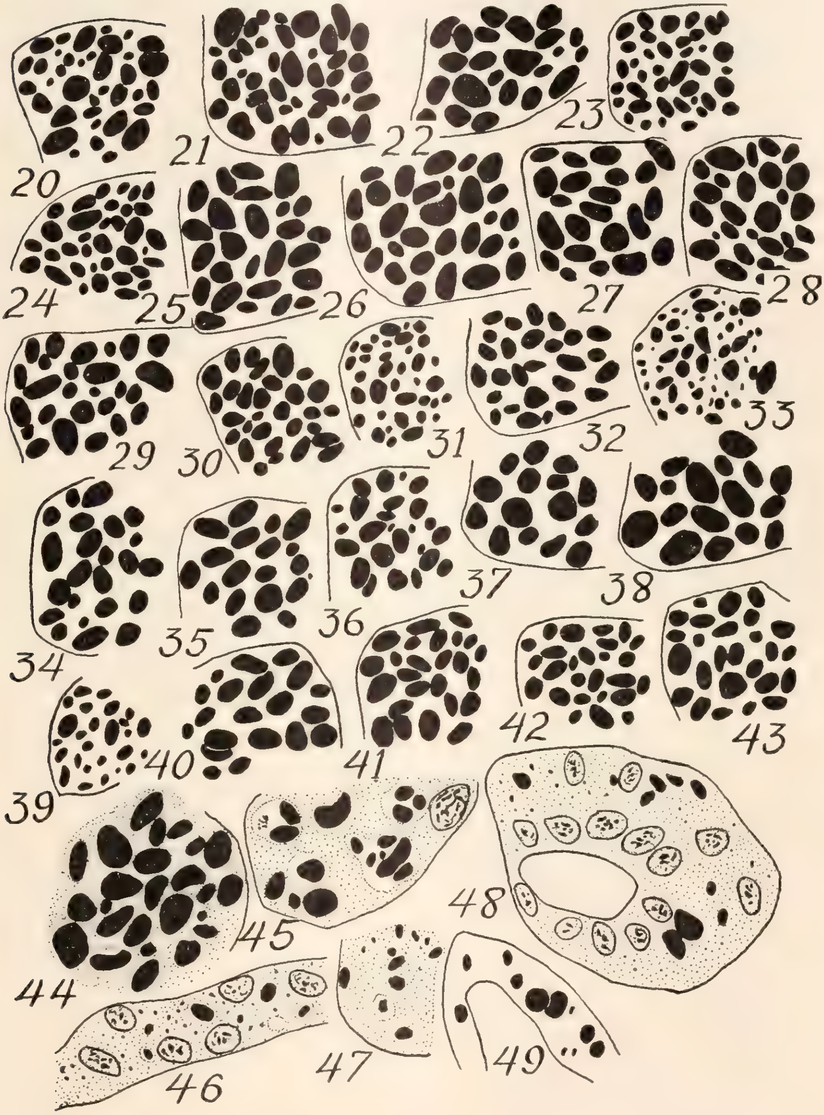
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Plate II. *Bufo cognatus*

These are (1) the micromeres which contain mostly small granules, (2) the macromeres which contain mostly large granules, and (3) cells located in a thick section between these which contain granules mixed and intermediate as to size. This last-mentioned region has been designated as the intermediate zone. These matters are illustrated in Figs. 1–3, 50–52, and 67–68.

I wish to call particular attention to the fact that it is not the absolute sizes of the yolk granules that is to be emphasized. Rather, it is the general appearance of the complex of granules in each type of cell. Some micromeres contain large granules intermingled with the smaller ones and the converse is true in the macromeres. The appearance is due in part to a greater number of the one type of granules or the other in any given cell and in part to the average sizes of the granules. The average length of the granules from the regions of the embryo of *Bufo cognatus* shown in Figs. 1, 2, 3, and the two portions of Fig. 7, for example, bear approximately the following relationships to one another: 1.0 : 1.2 : 1.6 : 1.1 : 2.2, the last two figures being for the dorsal lip of the blastopore and the adjacent yolk plug, respectively.

During gastrulation, the yolk granules maintain their original relationships as to size and appearance within each type of cell (Figs. 4–15). A striking contrast between the appearance of the complex of yolk granules in the dorsal lip of the blastopore and that in the cells

Explanation of Figures—Plate III

A. Blastula No. 1.2a.
    Fig. 50. Micromere.
    Fig. 51. Intermediate zone.
    Fig. 52. Macromere.
B. Neural plate stage.
    Fig. 53. Dorso-lateral endoderm.
    Fig. 54. Ventral yolk mass.
    Fig. 55. Dorso-lateral mesoderm.
    Fig. 56. Ventral ectoderm.
    Fig. 57. Neural plate.
    Fig. 58. Notochord.
C. Open neural groove.
    Fig. 59. Notochord.
    Fig. 60. Lateral ectoderm.
    Fig. 61. Neural fold.
    Fig. 62. Somite mesoderm.
    Fig. 63. Dorso-lateral endoderm.
    Fig. 64. Lateral mesoderm.
    Fig. 65. Ventral yolk mass.
D. Embryo just younger than that from which Figs. 59–65 were taken.
    Fig. 66. Posterior ventral yolk mass.
E. *Triturus torosus*, two-celled stage.
    Fig. 67. Yolk complex near the animal pole.
    Fig. 68. Yolk complex near the vegetal pole.

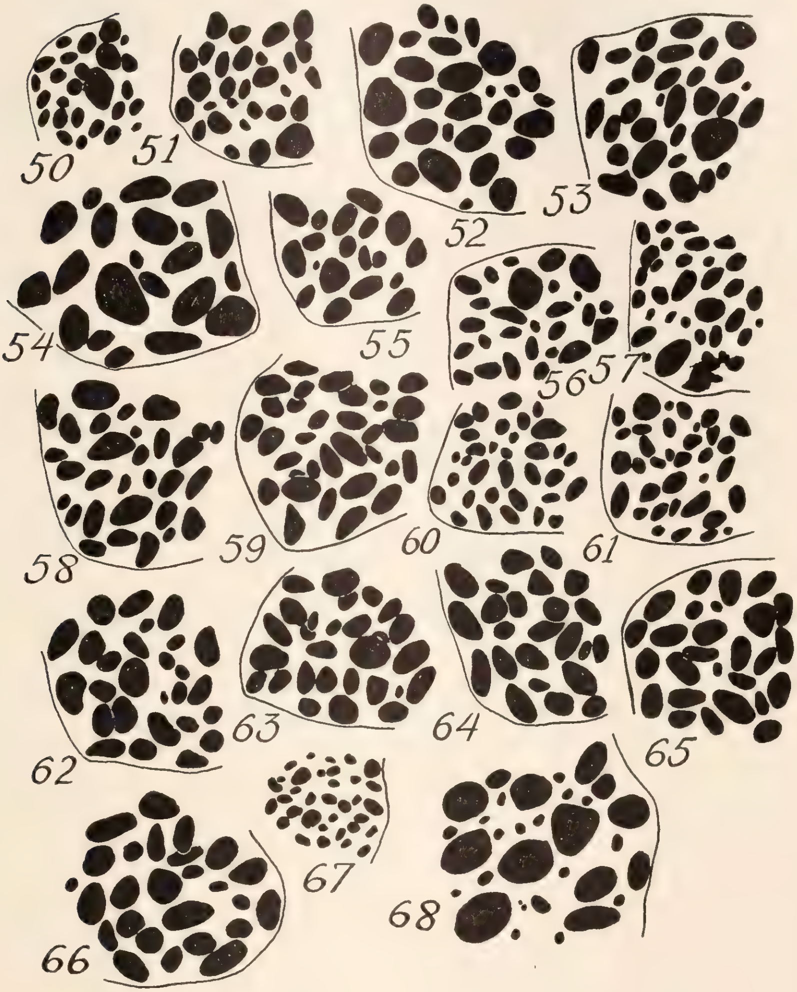


PLATE III. *Bufo woodhousii woodhousii* (except Figs. 67 and 68 which are of *Triturus*).

of the yolk plug just across the blastoporal groove illustrates this fact. (See Figs. 7 and 13.)

In later stages, the essentials in all of the species studied are also as earlier found for *Bufo cognatus*. The micromeres of the blastula pass under the dorsal lip of the blastopore for a short distance only, just enough to cover the yolk plug as the blastopore closes. The micromeres, therefore, give rise mostly only to ectoderm; mesodermal and endodermal derivatives contain only complexes of yolk granules characteristic of the macromeres of the blastula, except, possibly, some characteristic of the intermediate zone. (See Figs. 14–43 and 59–66.) Hence, it may be concluded without question that in *Bufo*, *Rana*, and *Scaphiopus* and probably also in *Triturus* (stages later than gastrulae not studied) the micromeres differentiate into ectoderm and the remainder of the embryo is derived from the macromeres and the cells of the intermediate zone. Since two orders, four families, and five species appear to agree so closely, it seems very probable that the principles here discussed will be found to apply generally to Amphibia.

In order to determine whether the embryo increases in size during early development, the measurements of embryos and larvae summarized in Table I were made. Taking change in diameter for stages through gastrulation and length thereafter as a measurement of growth, the figures show an increase between the early cleavage stages and the neural plate stage of 33 per cent and a width increase of 16 per cent. At the neural tube stage, the increase in length is 1 per cent more but the width has decreased 16 per cent. Between the neurula and the stage at hatching, the increase in length has reached 136.8 per cent of the diameter at early cleavage and the outer configurations of the embryo have become so irregular that exact measurements of width at any one level of the body-axis can have little meaning. A summary of these facts is presented in graphic form in Text-fig. 1.

It is well known that amphibian embryos absorb water during cleavage. Morgan (1906) found an increase in diameter of about 25 per cent between early cleavage and gastrulation in embryonic frogs, about one-half of which was due to the development of the blastocoel. The figure for *Bufo cognatus* is somewhat less than this (18.9 per cent), probably due to interspecific differences. However this may be, the increase in diameter is not too great to be accounted for almost or quite entirely by the absorption of water during cleavage, particularly if one consider the space occupied by the blastocoel. The data, therefore, confirm the earlier conclusion that no increase in protoplasmic mass occurs up to this stage, although the embryo does actually increase in size. Measurements of living embryos of *Rana sphenocephala* substantiate this general result.

In later stages, but prior to hatching, growth in length is quite rapid but the increase in width is not comparable. Cavities (archenteron, neurocoel, etc.) develop which take up space and the cells become progressively smaller, particularly in areas of high mitotic rate (Bragg, 1938; 1939). While by no means demonstrated, it seems very probable from these considerations that most of the increase in bulk prior to hatching takes place without material increase in fundamental protoplasmic constituents (except, of course, the ever present water). This conclusion, moreover, is strengthened by a study of the yolk granules in most regions of the embryo, the exceptions occurring during late embryonic life in those areas most active in differentiation.

TABLE I

Bufo cognatus. Growth of embryos and larvae as measured by length and width after preservation in 70 per cent alcohol. Measurements in mm. to the nearest 0.01. Animals grown in the laboratory at room temperatures (approximately 18–22° C.); cultures maintained in tap water with algae added as food after hatching.

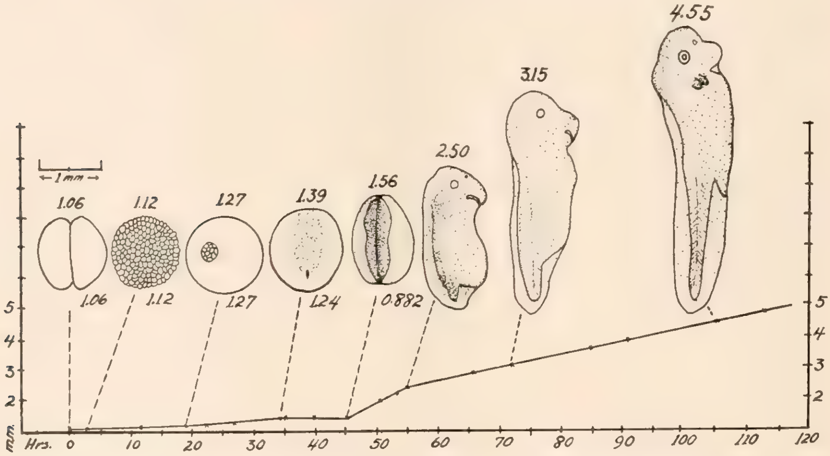
Stage	Age (hrs.)	No. used	Length	Width	Increase between stages		Total increase		Percentage of total increase	
					Length	Width	Length	Width	Length	Width
Early cleavage (2–8 cells)	1–2	20	1.06	1.06	—	—	—	—	—	—
Mid-cleavage	3–6	20	1.12	1.12	0.06	0.06	0.06	0.06	5.6+	5.6+
Gastrulation	18–20	20	1.26	1.26	0.14	0.14	0.20	0.20	18.9−	18.9−
Neural plate stage	33–35	20	1.41	1.24	0.15	−0.02	0.35	0.18	33.0+	16.0+
Neural tube stage	42–46	20	1.42	0.88	0.01	−0.36	0.36	−0.18	34.0−	−16.0+
Hatching	51–55	91	2.51	—	1.09	—	1.45	—	136.8−	—
Mouth a shallow pit	70–74	39	3.00	—	0.49	—	1.94	—	181.5+	—
Mouth a deep pit	74–100	20	3.15	—	0.15	—	2.09	—	197.2−	—
Mouth first functional	102–106	52	4.91	—	1.76	—	3.85	—	363.2+	—
Ready for metamorphosis	45 (days)	22	25.79	—	20.38	—	24.23	—	2285.8+	—

This is particularly true of *Scaphiopus:* for example, in the adhesive organ of this organism, the yolk granules of a late embryo are noticeably smaller than those in cells from which the anlage of the organ was derived. The same is true of the optic cup. But in the superficial ectoderm of the head and in the brain of the same embryo, they remain essentially unchanged.

From the foregoing observations, therefore, the following general conclusions may be stated:

(1) The embryo increases in size during all phases of development.

(2) This increase does not take place at a constant rate till hatching, after which it does so (at least as measured by length).



TEXT FIG. 1. Graph, increase in average length plotted against age in hours. The figures above the graph are camera lucida drawings, all to the same scale, of representative embryos and larvae of the stages indicated. The numbers above the drawings are the lengths (mm.) of the examples drawn; the numbers below are the widths of these same embryos.

(3) The increase up to the gastrula is due very largely, if not wholly, to the absorption of water, correlated with the space occupied by the development of the blastocoel.

(4) The development of the neurula from the neural plate stage is accomplished with little or no increase in bulk (length increases but width decreases).

(5) The most rapid growth in length occurs between the neurula and the stage at hatching; since the yolk is not altered within most of the cells during the greater part of this period, however, protoplasmic substance is increased but little in the embryo as a whole, even though the bulk of the embryo may increase due to the further absorption of water.

If the yolk is not utilized during early ontogeny, when and for what is it used? Observations upon late embryos and upon early to half-developed larvae of *Bufo cognatus* and *Scaphiopus* indicate the following: (1) The yolk granules begin to break up and to disappear in some regions before they do so in others. (2) Their digestion begins, in general, earliest in those embryonic regions which are first in histological differentiation. (3) Just prior to and during active digestion, vacuoles often appear in the cytoplasm and the yolk granules come to lie in these as though the vacuoles were formed around them: from this it is thought probable that the yolk is digested in intracellular vacuoles into which digestive enzymes pass from the cytoplasm much as in a protozoön.

Figures 30–49 illustrate these processes in *Bufo cognatus*. A cell of the optic vesicle in the neurula contains yolk granules comparable to those in the blastular micromeres (compare Fig. 1 with Fig. 30). In the ventral ectoderm of the same embryo (Fig. 31) they are smaller. In the adhesive organ (Fig. 33) they are not only smaller but also somewhat irregular in shape. The lateral ectoderm contains some irregularly shaped granules but the complexes of yolk in the mesoderm, endoderm, brain, and notochord remain unchanged. (See Figs. 32 and 35–38.) From this it appears that the yolk is used first in ectodermal structures, particularly those in the region of the anlage of the adhesive organ which is soon to differentiate and to function at hatching (Bragg, 1939*a*). In larval stages (Figs. 44–49) the yolk is disappearing in all regions except the ventral yolk mass.

Embryos of *Rana sphenocephala* show similar trends. In late embryos and early larvae, the yolk is beginning to be utilized in the brain, notochord, and the optic vesicle. The superficial ectoderm is probably just beginning to utilize the yolk but there has been no visible change in the mesodermal and endodermal portions. The more cephalic portions of the anlage of the central nervous system begin the use of the yolk before the more posterior portions. This illustrates the use of the yolk in correlation with anterior and cephalic differentiation in general as opposed to posterior and ventral differentiation.

These conceptions are further illustrated by the study of embryos and larvae of *Scaphiopus*. Little if any yolk is utilized before the neural tube is being formed. However, immediately after the neural tube closes, the differentiation of anterior and dorsal structures is well under way. This is especially noticeable in the adhesive organ but it apparently starts in the mesenchyme of the head before it does in the brain or superficial ectoderm in this species. The yolk granules in the notochord appear slightly decreased in size but those of the posterior and middle ectoderm, somite mesoderm, ventral yolk mass, and endoderm are still unchanged.

Just before hatching, the relation of the disappearance of the yolk to histological differentiation is still more striking. Ectodermal structures and some parts of the mesoderm are losing yolk but endodermal derivatives, for the most part, are not. The dorsal cephalic ectoderm, the optic cup, and the adhesive organ have lost more of the yolk than most of the other parts.

In a 78-hour larva (approximately nine millimeters in total length), differentiation has already reached a functional state in many organs. Some of the potential blood cells have no yolk granules whereas others have a few enclosed in vacuoles. Many contain small particles of yolk with no visible vacuoles around thèm and some have granules which are apparently unchanged. The superficial ectoderm in all parts of the body has lost much of its yolk. In one embryo, two cells were observed in this layer each of which contained a large vacuole in which were located small particles which stained like yolk. The endodermal wall of the gut still largely retains its yolk although a few of the granules are within vacuoles. The adjacent mesothelial wall of the splanchopleure has relatively few granules, some still quite large, others small. The myotomes of the tail are functional at this time. Sections of this region show the yolk to be small in amount and scattered. The cells of the ventral yolk mass contain granules of various sizes, but since some are definitely located in vacuoles, digestion of yolk has probably just begun in this region.

Discussion

In an earlier paper (1938) it was noted that the mitotic centers in the embryos of *Bufo cognatus* often do not correspond to the centers of susceptibility described by Bellamy (1919) in the embryonic frog and by Hyman (1921, 1926, 1927) and Rulon (1935) in other vertebrates. It was also noted that if the interpretation by these authors of the gradients of susceptibility as metabolic gradients be accepted, one seems justified in thinking of the regions of greatest susceptibility as regions where anabolic metabolism dominates katabolic metabolism. If this be granted, then it follows that histological differentiation is also dominated by anabolic as contrasted with katabolic processes, a conclusion in accord with the distribution of mitotic centers and with the *Gesetzmässigkeit* of Schmalhausen. The place where yolk first begins to disappear in the embryo (dorsal and cephalic regions, especially where most active differentiation is occurring) gives further evidence for this view, for some of these are the very regions which were found by others to be most susceptible to a variety of harmful influences; and they also tend to be the areas of lowest mitotic rate in certain stages (see especially Bragg, 1938, Table V, p. 165).

During cleavage, katabolic processes dominate and the respiratory relationships of the embryo require much oxygen (Bragg, 1939*a*). The source of the energy used during this period is still unknown, but the results of the study presented herein show clearly that the yolk is not used for this function (nor, indeed, for anything else) during this time.

At gastrulation, the metabolism of the embryo becomes differential, dominated in some regions by anabolism, in others by katabolism. This is shown both by the distribution of the mitotic indices at this stage and by the fact that a secondary center of susceptibility is set up in the dorsal lip of the blastopore (Bellamy, 1919). Since, however, the mitotic rate drops very suddenly at this stage, the embryo as a whole is likely dominated by constructive metabolism; but this is probably only another way of saying that the embryo enters the parabolic period of Schmalhausen. It seems probable, also, that early induction is anabolic in character, since the organizer of Spemann is located in the dorsal blastoporal lip and thus coincides with a center of susceptibility. This is, of course, only what one might expect, inasmuch as the fundamental function of induction seems to be the stimulation of cells to construct embryonic parts which would not arise, at least at a given time or place, without it. Furthermore, all of this correlates nicely with the distribution of the mitotic centers in the gastrula (Bragg, 1938, Table III, p. 161).

The time between early gastrulation and the formation of the neural tube seems to be one of great reorganization. The size of the embryo increases only insignificantly (Table I, Text Fig. 1) and the yolk remains inert; but in this short period (about twenty-five hours at ordinary temperatures in *Bufo cognatus* and probably even shorter in *Scaphiopus*), bilaterality is established, the notochordal and mesodermal anlagen make their appearance and the fundament of the whole central nervous system is formed. The distribution of the paths of cell-migrations and other morphogenic movements (Vogt, 1929 and others), as well as the places of greatest mitotic activity, seem best interpreted to mean that this reorganization is brought about almost wholly by cell-migration from specific centers of katabolic (mitotic) activity at specific places and times.

Following closure of the neural folds no further data on the mitotic indices in the amphibian embryo are available at the present time, but one would expect from the work on other forms that the mitotic rate in the embryo as a whole would progressively decline and that centers of high mitotic index would continue to arise, particularly just prior to the formation of specific anlagen (Derrick, 1937; Self, 1937; Jones, 1939). The rate of growth in length is greatly increased during the period between the neurula and hatching (a period of about ten hours in *Bufo cognatus* at room temperatures), but this involves the disappearance of the yolk from the cells only in the later stages (except in *Scaphiopus*). It seems probable, therefore, that the increase in mass is only slight and the apparent growth is due to the space taken up, in part by the development of cavities within the embryo, in part by decrease in width relative to length, and in part by further absorption of water. The yolk seems to be used at a slightly earlier period by *Scaphiopus* than in any of the other forms studied and this may be correlated with the exceptionally high rate of development which this form has (Trowbridge and Trowbridge, 1937; Trowbridge, 1939). However, even in *Scaphiopus* the yolk is used first by the regions of most active differentiation, mostly dorsal and cephalic in the embryo.

Late in embryonic life, the curve of growth becomes a straight line and from this time on the yolk progressively disappears from the cells, being used last in the large yolk mass ventral to the lumen of the gut.

Whether, in larval stages, the yolk is utilized primarily for histological differentiation or for increase in the bulk of the protoplasm could not be ascertained with certainty, since these two anabolic processes occur together. The methods used in this study could not, therefore, distinguish between them so far as their relations to the disappearance of the yolk is concerned.

SUMMARY

Sections of embryos and of larvae of several Amphibia, representing two orders, four families, and five species indicate that the yolk is carried passively in most cells till late in the embryonic period. Just before hatching in most species, but somewhat earlier in *Scaphiopus hammondii*, disappearance of the yolk begins in the areas of earliest histological differentiation, mostly dorsal and cephalic in the embryo. Since the regions of greatest susceptibility to injury reported by others are often the ones of lowest mitotic rate, it seems probable that histological differentiation is dominated by anabolic, rather than by katabolic, processes, just as growth must be. Similar reasoning shows that embryonic induction in the dorsal lip of the blastopore is also predominantly anabolic. This is indicated by the correlation

of the results of four methods of attack; the *Getzmässigkeit* of Schmalhausen, the mitotic index, studies of cell migration, and differential susceptibility to injurious environments, as reported by various workers, both in Europe and in America.

One interesting result for which no explanation is offered is that the yolk is not used during early ontogeny by any of the forms studied. This leaves no explanation for the source of the energy required by the very actively katabolic process of cleavage. Little if any increase in protoplasmic mass occurs before the yolk begins to be utilized. It is still uncertain whether the yolk serves primarily for increase in protoplasmic mass, for histological differentiation, or for both, since these predominantly anabolic processes proceed concurrently in the late embryonic and larval periods. However, since the process of early embryonic organization and the laying down of most of the fundaments of the major organ systems occur before the yolk is used, it is clear that all of the early morphological manifestations (whatever their individual natures) proceed normally without the aid of the yolk.

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FURTHER EXPERIMENTS ON THE DECOMPOSITION AND REGENERATION OF NITROGENOUS ORGANIC MATTER IN SEA WATER[1]

THEODOR VON BRAND, NORRIS W. RAKESTRAW
AND CHARLES E. RENN

(*From the Woods Hole Oceanographic Institution, Woods Hole, Mass.*)

In an earlier paper we reported (1937) that the cycle of decomposition and regeneration of nitrogenous organic matter in sea water can be reproduced experimentally. The main stages in this cycle are: living organism—dead organism—ammonia—nitrite—nitrate—living organism. In view of the importance of nitrogenous material in the economy of the sea, it seemed worth while to carry these experiments somewhat further, and especially to consider the following questions:

1. Is it possible to reproduce more than one cycle in the same water?
2. Can the cycle be made shorter by eliminating certain stages?
3. Do successive cycles differ significantly in character or in rates of development?
4. In what ways do anaerobic and aerobic decomposition differ?
5. How completely can the changes observed in the different forms of nitrogen be accounted for in terms of each other; in other words, how constant is the quantitative balance?

METHODS AND MATERIAL

The plan of the experiments was the same as in our previous investigation. Organic material was suspended in sea water in large carboys and allowed to decompose in the dark, during which time chemical analyses were made periodically. Artificial cultures of *Nitzschia Closterium* were chosen as a source of organic matter, for our previous experience had shown these diatoms to be more satisfactory from an analytical standpoint than mixed plankton. Waksman, Stokes and Butler (1937) also used them successfully for a somewhat similar purpose. The diatoms were separated from the culture medium by centrifugation, washed several times with nitrate-free sea water, and finally suspended in a carboy of sea water which had been filtered through No. 4 sintered-glass. All experiments were carried out at the uncontrolled room temperature, varying from 15° C. in the winter to 25° in the summer. Before samples were removed for analysis the carboys were shaken vigorously to distribute the suspended matter evenly. When the latter showed any tendency to stick to the glass it was loosened with a rubber-tipped glass rod before shaking, after the rubber had been carefully cleaned to avoid contamination.

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[1] Contribution No. 222 from the Woods Hole Oceanographic Institution.

The methods for the determination of particulate nitrogen, ammonia, nitrite and nitrate were the same as those used in our previous experiments and have already been described in detail.

The Possibility of Consecutive Cycles

In our previous investigation we found that the nitrate resulting from plankton decomposition could be regenerated into diatom protoplasm. This raised the question of the possibility of repeating such a cycle of decomposition and regeneration more than once in the same water. When the original plankton has undergone decomposition and the nitrate stage has been reached the water is inoculated with fresh *Nitzschia*. After about a week in the light an abundant growth is always observed. The nitrate drops to a minimum and when placed in the dark this newly-developed plankton undergoes decomposition again. In Series 12 three complete, successive cycles were carried out in this way. In another similar series, for which the data are not given, two cycles were completed, and in several others a part of the second cycle.

A poor diatom growth was observed after the second cycle in Series 12 (Table I), in which unidentified algae developed, among the *Nitzschia*.

It was important to know if a regeneration of phytoplankton material is possible in parts of the decomposition cycle other than the nitrate stage. During the plankton decomposition in Series 23, when the ammonia had reached its maximum, but before nitrite or nitrate had appeared, a portion of the water was transferred to another carboy (23A). After inoculation with *Nitzschia* and a week's exposure to light the ammonia had almost entirely disappeared and a heavy diatom growth had occurred. In a similar way Series 19A was removed from No. 19 before the nitrate had reached its maximum. Here, too, diatoms developed rapidly and abundantly and the soluble nitrogen compounds disappeared almost quantitatively. At these stages, at least, no toxic substances had been formed, or deficiencies developed, which could inhibit the development of phytoplankton. "Short-cuts" in the nitrogen cycle can evidently take place, and the possibility of their occurrence in nature suggests an explanation for

TABLE I

Series 12. Woods Hole harbor water. Fresh culture of *Nitzschia Closterium.* Quiet, in dark. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate + Nitrite	Nitrate	Total nitrogen detd.	Diatom count $\times 10^3$ per ml.
7- 5-37	256	31	0	14	14	301	174
7	225	65	0	14	14		134
11	107	215	0				12
17	92		0	19	18		3
23	78		0	11	11		<1
30	49	260	0	11	11	320	<1
8- 5	46		1	11	10		<1
13	45		5	25	19		<1
20	40	210	105	110	5	360	<1
24		70					
27	41	30	265	365	0	435	<1
9- 2	31	13	350	355	5	402	
12			390	395	5		
25		25	370	390	20		
10-28	37	61	0	305	305	413	
31	Reinoculated with diatoms and put in light						
11- 7							137
13							780
16	455	21	0	2	2	478	
16			Put in dark				
21							706
12- 1	164						40
24		59	0				
2- 1-38			1	8	7		
4		247					
24	189	246	1			(440±)	
5- 6		0	70	350	280		
29			0				
6-25	142	12	1	300	300	455	
7- 5	Reinoculated with diatoms and put in light						
12	408			80	80?	(490±)	
15				10			
18	489	0	0	40	40	529	
18			Put in dark			*	
29	304	115	0	25	25	445	
8- 5	190	175	4	18	14	387	
12	202	110	45	50	5	362	
19	196	20	150	155	4	371	
25	208	10	170	160	0	388	
31	237	0	200	(160)	0	437	
9-13	260	18	0	300?	300?	578	

* Dissolved organic nitrogen = 370.

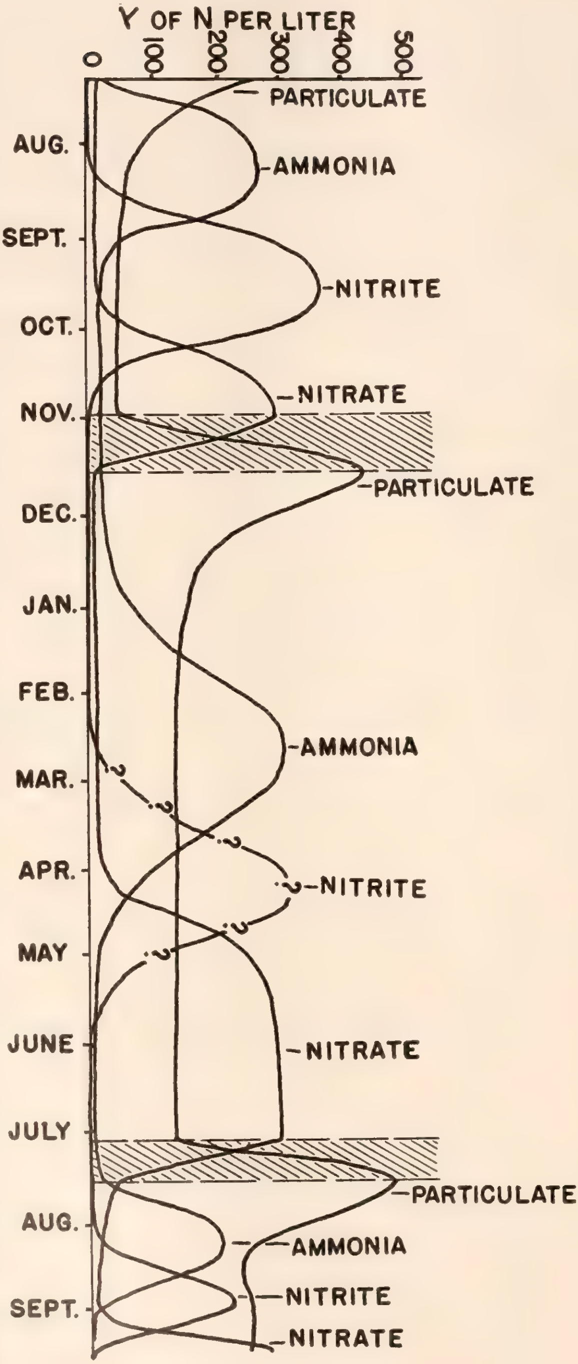


Fig. 1. Series No. 12. The disappearance of particulate nitrogen and the simultaneous changes in ammonia, nitrite and nitrate, plotted against time. Source of organic matter, fresh culture of *Nitzschia Closterium* suspended in sea water from Woods Hole harbor. The shaded areas represent regeneration periods, with the culture in the light; the remainder, decomposition in the dark.

the rapid succession of great numbers of phytoplankton organisms belonging to different species. In nature, all stages of the cycle must be taking place simultaneously, and the momentary picture is simply one of equilibrium.

Chemical Observations

Decomposition of Particulate Nitrogen

The particulate nitrogen is that contained not only in diatoms, but also in bacteria and miscellaneous debris. This material began to decompose rapidly in all cases, as soon as the water was placed in the dark. It never disappeared entirely, however, but reached an apparently constant level after periods varying from two to six weeks. This was true not only in the first decomposition cycle but also in the subsequent ones. This residual material is very resistant to further decomposition, and consequently the level of residual particulate nitrogen is higher after each successive decomposition cycle. In the first cycle of Series 12, for example, the residual particulate nitrogen was about 40 γ per liter, in the second cycle about 150 γ, and in the third 200 γ. These amounts are about 16 per cent, 33 per cent and 41 per cent of the particulate nitrogen present at the beginning of each respective cycle. It seems not unlikely that under natural conditions in the sea the plankton is incompletely decomposed, and a large part of the particulate nitrogen found by von Brand (1938) in the deeper levels may be contained in such resistant or slowly decomposing plankton and bacterial residues. The occurrence of bacteria and debris in this resistant fraction is indicated by the fact that the quantity of particulate nitrogen does not consistently follow the diatom count. While the sum of particulate and ammonia nitrogen is fairly constant during the first part of the decomposition cycle, the diatom count falls off much more rapidly than does the particulate nitrogen. Nevertheless, in nature this refractory residue cannot be entirely resistant to decomposition, otherwise the insoluble nitrogenous material, in the water or on the bottom, would increase without limit.

A curious irregularity was observed in Series 25, which was aerated by a constant stream of pure air. After about six weeks of normal decomposition, accompanied by the appearance, first of ammonia and then of nitrite, the particulate nitrogen rose abruptly to its initial value and the ammonia and nitrite disappeared entirely. A microscopic examination, carried out for us by Dr. Lois Lillick, showed the presence of an enormous number of bacteria and a few flagellates. The diatoms had disappeared completely. This phenomenon must be attributed to the development of a peculiar bacterial flora, since we did not observe it in any other series, including No. 24, which contained the same water and plankton as No. 25 but differed only in not being agitated with an air-stream during the decomposition.

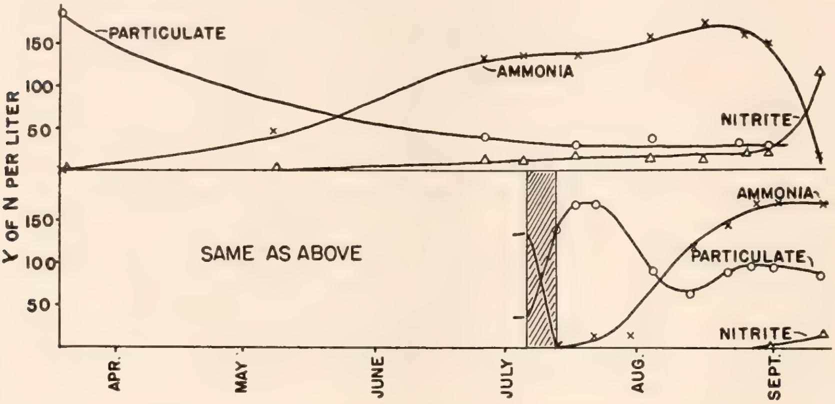
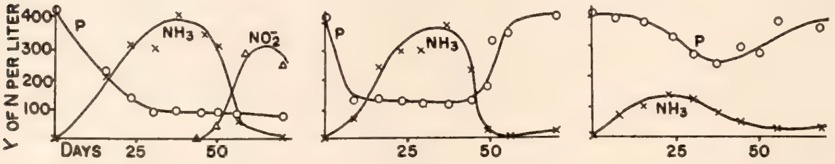


FIG. 2. Series No. 23 (above) and 23A (below). The disappearance of particulate nitrogen and the simultaneous changes in ammonia, nitrite and nitrate, plotted against time. Source of organic matter, fresh culture of *Nitzschia Closterium* suspended in sea water from the Sargasso Sea. The shaded area represents a regeneration period, with the culture in the light; the remainder, decomposition in the dark.



Series No. 24 Series No. 25 Series No. 26

FIG. 3

The disappearance of particulate nitrogen (*P*) and the simultaneous changes in ammonia and nitrite in cultures of *Nitzschia Closterium* in sea water from Woods Hole harbor. The three cultures were identical at the start. Series No. 24 stood quietly in the dark. Through No. 25 a continuous stream of purified air was bubbled. The air was completely removed from Series No. 26 and a continuous stream of purified hydrogen bubbled through it.

Ammonia

During the first decomposition cycle, in all cases, ammonia appeared in the water rapidly and in such amounts as to exclude the possibility of soluble nitrogen compounds intermediate between particulate nitrogen and ammonia. This but confirms our previous findings. But in the second cycle, although the particulate nitrogen disappeared at about the same rate, ammonia was not formed as rapidly as in the first. In Series 19A, for example, ammonia appeared only after 30 days of decomposition in the dark, during which time the particulate nitrogen had diminished by 160 γ per liter. The same behavior is found in Series 23A and in the second cycle of No. 19, and very likely also in the second cycle of Series 12, although the data are incomplete in the latter. These cases indicate the formation of soluble nitrogen compounds of higher molecular weight, intermediate between dead protoplasm and ammonia. Although there are two or three possible explanations for this lag in ammonia formation, we are not yet inclined to urge the acceptance of any one of them. It is also to be observed that the third cycle of Series 12 resembles the first in its more rapid rate of ammonia appearance.

In the first cycle it generally required from 16 to 25 days for the ammonia to reach its maximum, where it remained for a period of from 21 to 50 days before dropping. A notable exception, however, occurred in Series 22 and 23, in which the ammonia did not appear until about the forty-eighth day, required three to four months to reach its maximum, and remained for another two months before disappearing entirely. This unusually long duration of the ammonia stage would seem to be connected with the source of the water in the experiments. That used in Series 22 came from the Caribbean, and that in Series 23 from the Sargasso Sea, while in all other cases the water was taken from near the shore. Since the diatoms in all experiments were from persistent cultures the bacterial flora introduced with them was presumably constant, but whether the difference in decomposition is due to differences in the bacterial flora of the water itself or to such properties of the water as might influence the growth of bacteria, is not yet clear.

The lag in the oxidation of ammonia to nitrite in Series 22 and 23 was apparently not due to the absence of the necessary bacteria, for portions of these cultures were inoculated with 1 ml. from Series 12, in the midst of its nitrite stage, without any resulting change in the rate of nitrite formation.

Nitrite

In the first cycle nitrite began to appear in 31 to 58 days, corresponding to the beginning of the disappearance of ammonia. It reached its maximum when the ammonia had dropped to a minimum; that is, in a period of from 46 to 74 days. The total duration of the nitrite stage of the first cycle was quite irregular: two months from beginning to end in Series 12, but more than six months in Series 13 (See Table II). The lag in the latter case may in some way be related to the continuous aeration.

Our data concerning nitrite in the second cycle are too scattered to tell whether or not the rate of nitrite formation is the same in the second cycle as in the first. In the third cycle of Series 12 nitrite, like ammonia, developed rapidly.

TABLE II

Series 13. Woods Hole harbor water. Fresh culture *Nitzschia Closterium* in dark, with ammonia-free air bubbling through. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate + Nitrite	Nitrate	Total nitrogen detd.	Diatom count $\times 10^3$ per ml.
7- 8-37	269	43	1	11	10		234
10	380	11	0	17	17	407	274
13		115	0				
14	280						74
19	109	240	0	11	11	360	2
24	86	300	0	11	11		<1
31	60	310	0	11	11	381	<1
8- 5	53		1	11	10		<1
13	29		0	11	11		<1
20	25	400?	0	9	9	434	<1
24		360					
27	23	330	3	10	7	365	<1
9- 2	34	350	30	35	5	419	
12			330	340	10		
25		15	330	350	20		
10-28	7	82	345	(310)	?		
11-18			321				
2- 1-38		20	272	(260)	?		
24	74	20	232				
5- 6			0	400?	400?		
6-25	120	25	1				
7-15	118	74	7	230	225	422	
7-18		Put in light, not reinoculated					
8- 2	97	20	4	200	200		<1
4	Reinoculated with diatoms						
10	389	9	0	10	10	408	*

* No diatoms present; unidentified algae.

Nitrate

Nitrate begins to appear only when nitrite disappears, and this never seems to happen as long as a significant amount of ammonia remains. Since one cannot rely upon a greater accuracy than 10 per cent in the analytical determination, any quantitative balance is uncertain when large amounts of nitrate are involved.

TABLE III

Series 19. Woods Hole harbor water, collected 2–25–38. Fresh culture *Nitzschia Closterium.* Standing quiet, in dark. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate + Nitrite	Nitrate	Total nitrogen determined	Diatom count $\times 10^3$ per ml.
2–25–38	316						
26		12	0	10	10	338	275
5– 6		41	12	25	13		
6–25	71	50	87	285	200	406	
7– 6		5	12		(Series 19A separated)		
15	48	15	1	350	350	414	
7–18	Reinoculated with diatoms and put in light						
29				17			
8– 2	396	0	0	10	10	406	56
8– 2	Put in dark						
10	451	0	0	15	15	466	240*
16	262	10	0	20	20	292	220†
23	201	30	0	15	15	246	
30	304?	15	1	20	19	339	
9–13	193	70	0				

Series 19*A*. Portion separated from Series 19 on 7–6–38. Reinoculated with diatoms and put in light.

Date	Particulate	Ammonia	Nitrite	Nitrate + Nitrite	Nitrate	Total nitrogen determined	Diatom count $\times 10^3$ per ml.
7– 6		5	12				
12	382	12	0	15	15	409	475
12	Put in dark						
18		0	3	25	22		350
21	396	7	0	10	10	413	
29	252	5	0	15	15	272	5
8– 5	221	90	0	15	15	326	
12	141	175	0	10	10	326	
20	208	186	0	10	10	404	
25	187	250	0	15	15	452	
31	201	250	0	20	20	471	
9–13	171	250	0	10	10	430	

* Few *Nitzschia;* mostly *Skeletonema Costatum.*
† Both *Nitzschia* and *Skeletonema.*

ANAEROBIC DECOMPOSITION

In addition to the experiments already described, which were carried out under aerobic conditions, the anaerobic decomposition of diatoms was also studied in two series. The carboys containing the water and diatoms were first evacuated to remove all the air from the water and the container. Then a slow, continuous stream of purified hydrogen was bubbled through the water. After decomposition in the dark for some time a strong odor of hydrogen sulfide was observed. In both cases the particulate nitrogen diminished very slowly, but remained constant at a level very much higher than that in the aerobic decompositions. The diatom counts also remained high; in fact, when Series 26 was discontinued, after 10 weeks, living diatoms were still found, which grew when placed in fresh culture medium.

TABLE IV

Series 22. Water from the Caribbean Sea. (18°–35′ N; 79°–14′ W); one year old. Fresh culture *Nitzschia Closterium*. Quiet, in dark. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate + Nitrite	Nitrate	Total nitrogen determined	Diatom count × 10^3 per ml.
3–18–38	123	0	0			125*	213
5– 6		25	0	10	10		
6–25	41	113	12	10	0	166	
7– 6		115	12				
13	22						
15		118	12	25	13		
8– 3	30	150	11	12	1	191	
13		145	12		(Series 22B separated)		
23	23	150	16	20	4	193	
30	30	125	30	30	0	185	
9–13		20	90			(150±)	

Series 22*B*. Portion separated from Series 22 on 8–13–38 and inoculated with 1 ml. from Series 12. In dark.

8–13		145	12				
20		205	22	25	3	160	
30		210	36	35	0	245	
9–13		220	50			(270±)	

* Dissolved organic nitrogen = 93.

Ammonia also increased in this series for the first three weeks, and then gradually disappeared. In the other series no appreciable amount of ammonia was formed; on the contrary, a small amount of that originally present disappeared. This ammonia was not recoverable from the effluent hydrogen, nor are we able as yet to account for the behavior of ammonia in either of these series. As might be expected, no nitrite was formed.

After about two months under anaerobic conditions a portion from one of the cultures was aerated and kept henceforth aerobically.

Two months later a large amount of ammonia had been formed, but no nitrite or nitrate had appeared by the time the experiment was stopped.

Quantitative Balance

In our previous investigation we pointed out that in nearly every case studied the total determined nitrogen in the system (that is, the sum of the particulate nitrogen, ammonia, nitrite and nitrate) increased continuously throughout the period. In the cases we are now reporting the quantitative balance is much more satisfactory. In Series 13, 22, 23, 24 and 25 the changes in total nitrogen are small and probably explainable in terms of accumulated errors. In Series 12, 19, 19A, 23A and one other, the increase observed is too large to be accounted for in this way, but is less, relatively, than the increase noted in our first experiments. Previously, we discussed three possible explanations for this increase in total nitrogen: systematic errors in the determination of particulate nitrogen, the participation of dissolved organic nitrogen in the decomposition, and nitrogen fixation. We were in no position to prefer any one of these explanations. The fact that a good nitrogen balance was observed in half of our later experiments, including one which extended over more than a year, seems definitely to eliminate the possibility of systematic errors. The difficulty of determining dissolved organic nitrogen with any accuracy makes it almost impossible to test the second hypothesis directly. (The one determination given in the data for Series 12 was made, with some difficulty, by the method of Krogh, 1934.) However, we sought to investigate this question indirectly, by trying to see whether ammonia appears, on standing, in water devoid of gross particulate matter, and if so, whether the process is related to the content of dissolved organic matter. Two filtered samples of water, one from Woods Hole harbor and one from the Sargasso Sea, were placed in the dark and the usual determinations made periodically. Previous work had shown that the harbor water had a higher organic nitrogen content than water from the open sea, but there was no difference in the behavior of the two kinds of water. A small increase in nitrogen, about 60 γ per liter, was observed in both. This could indeed be the result of the participation of dissolved organic nitrogen, but we are inclined to believe that it is due to an entirely different cause. All the stored carboys were tightly stoppered, but before the removal of samples they were vigorously shaken and opened. During this time the water comes into contact with a rather considerable quantity of air, from which it may take up ammonia. To test this possibility, clean filtered air was aspirated for 12 hours through a sample of sterilized Sargasso water. This resulted in an increase of 50 γ of ammonia-N and 20 γ of nitrate-N. Such a quantity of ammonia is not surprising, in view of the amount of decomposing organic matter in this laboratory and its vicinity. It seems quite possible, therefore, that at least a large part of the increase in total nitrogen observed in some of our experiments may be due to contamination from the air. Nevertheless, this is not conclusive, and we are still in no position to exclude the possibility of either nitrogen fixation or participation of dissolved organic nitrogen. Atmospheric contamination was ruled out in Series 13 and 25, through which purified air was aspirated, and in these cases, indeed, the total nitrogen did not increase. Still, no increase was observed in Series 22, 23 and 24, which were not aerated, but which stood side by side with carboys in which nitrogen accumulated. Further work is still being done in the effort to clear up these discrepancies.

Summary

1. Several consecutive cycles of decomposition and regeneration were carried out in the same water.

2. It is confirmed that in the first cycle the main stages of decomposition are: dead body—ammonia—nitrite—nitrate. In the second cycle there is evidence of intermediate soluble substances between dead body and ammonia.

3. Under anaerobic conditions the initial states of decomposition take place more slowly than under aerobic conditions, and no nitrite or nitrate is developed.

4. Regeneration of nitrogen into phytoplankton protoplasm is possible not only in the nitrate stage but also in the ammonia stage and before the nitrate has reached its maximum.

5. The quantitative nitrogen balance was better than that reported in previous experiments, and possible reasons for the discrepancies still present are discussed.

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PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1939

July 5

The effect of biologically conditioned medium upon the growth of Colpidium campylum. G. W. Kidder.

The effects of metabolic products upon a population has been the subject of many investigations in the past. Decreased growth rate and population yield due to the accumulated products of metabolism have been reported for many organisms, especially micro-organisms. On the other hand, growth acceleration has been noted in many instances and the effect ascribed to substances given off by like or different species.

Recently we have been interested in making comparisons between some of the phases of protozoan growth and those which have been reported for the bacteria and yeasts. In these studies it has been possible to utilize many of the techniques employed by the bacteriologist due to the fact that the species of protozoa used were all bacteria-free and growing in non-particulate broth. The following results were obtained from studies on the ciliate *Colpidium campylum.*

Manipulation of the conditioned media (2 per cent proteose-peptone-1 per cent dextrose broth) was as follows: *control*—plain, fresh broth; *conditioned*—broth in which *Colpidium* had grown for varying lengths of time and the ciliates centrifuged out; and *filtered conditioned*—conditioned medium which had been passed through a Seitz bacteriological filter. All experiments were performed with the three types of media run in parallel series, at 26° C.

Growth in control flasks (inocula taken from the log phase) exhibited typical logarithmic growth for 28 to 40 hours, depending upon the size of the inoculum. After a very short "negative growth acceleration" period the curve tends to level off and remains at a relatively high level for some weeks. Medium conditioned 60 hours and then inoculated with log ciliates shows a large and significant acceleration during the early growth period. Negative growth acceleration occurs sooner than in the control and the maximum yield is never as great. Filtered medium which has been conditioned 60 hours produces a decided lag phase, indicating inhibition in the early periods of growth. Maximum yield is similar, however, to that in the conditioned (supernatant of a centrifugate). Increasing the length of the conditioning period lowers the curves for both the conditioned and the filtered conditioned media. Slight acceleration in the former was obtained, however, after 4 weeks of conditioning, while the lag period of the latter was increased. Acceleration over the growth of the control was not found after 8 weeks of conditioning although the difference between this curve and that produced in the filtered 8 weeks conditioned was still of the same magnitude as those previously described.

From the results of these experiments it might be said that it appears that substances are released into the medium by growing ciliates which produce a decided effect upon subsequent growth of fresh ciliates. We can think of these substances as falling into two categories, one a growth inhibitor which will pass through an asbestos filter and the other a growth accelerator which will be absorbed on the filter. When both are present together (as in the supernatant of a centrifugate) the accelerator masks the effect of the inhibitor during the early growth phases. When the accelerator has been removed (filtrate) the inhibitor action is marked. There appears to be a differential as to time of production as the accelerator becomes increasingly less effective as time of conditioning increases while the inhibitor becomes more effective. Until more work is completed upon these very interesting substances the above analysis can only be a conjecture.

Respiration in Chilomonas paramecium. John Hutchens.

The rate of consumption of oxygen and the respiratory quotient of chilomonads grown in sterile, pure, mass cultures in a medium containing CH_3COONa, NH_4Cl, $(NH_4)_2SO_4$, K_2HPO_4, $MgCl_2$, and $CaCl_2$ were measured at 25° C., using simple Warburg respirometers. The rate of consumption of oxygen was found to vary inversely with the age of the culture from which the organisms were taken, and also with the strain of chilomonads used. The respiratory quotient was found to vary directly with the rate of oxygen consumption, *i.e.*, inversely with the age of the culture. The following table compares the rate of oxygen consumption and the respiratory quotient of chilomonads from two different strains at different times following inoculation of the cultures.

The relation between the rate of consumption of oxygen by chilomonads and the respiratory quotient

Age of culture in hours	Strain of chilomonads	Rate of consumption of oxygen in cu. mm. per 10,000 chilomonads	Respiratory quotient
24	2	0.40	0.93
24	1	0.35	0.91
48	2	0.24	0.80
48	2	0.23	0.81
48	2	0.23	0.79
48	1	0.17	—
72	2	0.17	0.75
72	2	0.17	0.74
72	1	0.12	

Temperature and starch and fat in Chilomonas paramecium. Jay A. Smith.

Starch and fat in *Chilomonas* are contained in particles, and by proper staining (Lugol's solution for starch and Sudan III for fat) the size of each particle and the total volume of each substance can be ascertained.

Temperatures from 9.5° C. to 39° C. were used.

The solution, which contains inorganic salts and sodium acetate, was that employed by Mast and Pace and co-workers.

Within viable temperatures, it was found that the volume of starch remains constant, that the volume of fat decreases as the temperature rises, but that the volume of both starch and fat synthesized by the progeny of one chilomonad during a period of 24 hours increases greatly. This indicates that the rate of synthesis within the viable temperature range may be the factor that controls the frequency of division.

At a lethal low temperature (9.5° C.) there is no division, the volume of starch gradually decreases, but the volume of fat increases, which indicates that in the absence of synthesis of starch, starch is transformed into fat.

At lethal high temperatures (32.5°–39° C.) the frequency of division decreases as the temperature rises, and there is an accumulation of starch and fat that reaches a maximum at 35° C. and then decreases. This indicates that the rate of synthesis of starch and fat increases in the same manner that it did at viable temperatures, and thus the accumulation of starch and fat is due to two interacting factors: the rate of synthesis and the period the chilomonads live.

Thus, the decrease in the frequency of division at lethal high temperatures is probably caused by the same factors that cause death, but there is no relation between the death, the frequency of division *and* the rate of synthesis of starch and fat.

JULY 11

The differentiation of isolated rudiments of the Amblystoma punctatum embryo. Floyd Moser.

Using the technique which has been developed in Harrison's laboratory, a number of structures associated with the embryo of *Amblystoma punctatum* have been isolated and cultured *in vitro* under nearly aseptic conditions. The embryos were generally in Harrison's Stage 29 at the time of operation, though in some cases Stages 27, 28 and 30 were also used. Among the structures isolated were the balancer rudiment, gill rudiment, limb rudiment and tail bud. During the 10 or 11 stages immediately following operation the explants keep pace both in rate and degree of differentiation with the intact structures in unoperated control animals. This, while it is itself of some interest, takes on more special significance in view of the fact that the explants are entirely free of nervous and vascular connections. The tail bud explants alone are exceptions to the latter statement in that they doubtless are well provided with nervous structures.

Typically, the isolation of the ectoderm of the gill rudiment yielded nothing that was gill-like in appearance, while the ectoderm plus the underlying layers of mesoderm gave rise to a single gill. Isolations of gill rudiment consisting of ectoderm, mesectoderm, mesoderm and entoderm gave rise to three gills, which in external appearance, were not unlike those of the unoperated control animals.

Explanted balancer rudiment consisting of ectoderm and the underlying layers of mesoderm and entoderm gave rise to what appeared to be perfect balancers, but these were no better than those obtained from isolated balancer ectoderm alone.

Limb rudiment isolations consisted of the mesoderm and its overlying sheet of ectoderm. As differentiation of the explanted structure takes place, it becomes possible to tell whether the rudiment has come from the right or the left side because of the presence of characteristic surface contours.

When the unoperated controls reach Stages 38 to 39, the isolated tails exhibit function in the sense that they twitch when stimulated by means of slight pressure with a hair-loop.

The present experiments, as well as others involving the fragmentation and fusion of rudiments, and experiments in which isolated rudiments of various ages have been grafted back to host embryos, will be reported at greater length elsewhere.

The production of duplicitas cruciata and multiple heads by regeneration in Planaria. Robert H. Silber.

The observations reported were taken from a paper published in *Physiol. Zool.* (vol. 12, No. 3, July, 1939, p. 285) by Robert H. Silber and Viktor Hamburger and entitled: "The production of duplicitas cruciata and multiple heads by regeneration in Euplanaria tigrina."

Neural differentiation without organizer. L. G. Barth.

Previous experiments have shown that the amphibian ectoderm in the gastrula stage may very easily be stimulated to form a neural plate. This suggested that under certain conditions the ectoderm might form a neural plate without the organizer or any external stimulus. It was first found that the fusion of two to eight explants of ectoderm would develop with neural tubes, while a single explant formed only a mass of epidermis.

Following this a number of fusions between two pieces were made, some with the antero-posterior axis coincident and some reversed. When the antero-posterior axis of the two explants coincides a neural tube differentiates but when the axes are reversed only epidermis results. Thus the polarity of explants must be preserved in order that a neural tube may form without an organizer.

The neural tube differentiates from the anterior end of the explant since when two explants are united by their anterior ends a neural tube appears in the middle. Further when an anterior half explant is fused with the anterior end of a whole explant the neural tube forms at the end. Other fusions also show that the anterior end forms the neural tubes. There is then an antero-posterior polarity or gradient in the isolated ectoderm and this polarity must be maintained in order to obtain neural tubes without organizer.

This antero-posterior gradient exhibits itself further by differences in oxygen consumption. The roof of the blastocoel was cut into four parts along the antero-posterior axis from the dorsal lip to the ventral epidermis. The results show that the oxygen consumption is high in the anterior pieces of ectoderm and low in posterior pieces. The dorsal lip respires at about the same rate as the anterior end of the ectoderm.

July 18

The effect of substrate concentration on the cyanide sensitivity of the oxygen consumption of yeast. Kenneth C. Fisher.

This paper has already appeared in the *Proc. Am. Physiol. Soc.* under the title, "The sensitivity of the oxygen consumption of yeast to cyanide" (*Am. Jour. Physiol.*, **126**, pp. 491–492).

A comparison of cyanide and azide as inhibitors of cell respiration. C. W. J. Armstrong.

This paper was published by C. W. J. Armstrong and Kenneth C. Fisher in the *Proc. Am. Physiol. Soc.* under the title: "The effect of sodium azide on the frequency of the embryonic fish heart" (*Am. Jour. Physiol.*, **126**, p. 423).

The relation of blood to the respiratory ability of fresh water fish. Laurence Irving.

Fresh water which is habitable for fish may be well or poorly oxygenated and may contain CO_2 at practically zero or at a quite noticeable pressure. The properties of the blood for transport of oxygen also vary. Oxygen dissociation curves for the blood of seven common fish at 15° in the absence of CO_2 are similar and differ only in ease of combination with oxygen. The P_{O_2} necessary for half saturation defines the position of the curve for each fish and is as follows: catfish 1.4 mm.; carp 5 mm.; bowfin 4 mm.; common sucker 12 mm.; brown trout 20 mm.; brook trout 22 mm.; rainbow trout 22 mm.

Carbon dioxide increases the P_{O_2} necessary for oxygenation. If the increase in P_{O_2} for half saturation be divided by the P_{CO_2} which effects that change, then the quotient distinguishes the blood of each fish. The quotients are: catfish 0.3; carp 1.0; sucker 3.5; brook, brown and rainbow trout 6.0. The effect of CO_2 on half saturation with oxygen indicates the influence upon unloading of oxygen from blood into the tissues.

Carbon dioxide also reduces the ability of blood to combine with oxygen at 150 mm. pressure of oxygen. The curves relating oxygen contained at $P_{O_2} = 150$

mm. with increasing P_{CO_2} are similar for the various fish and reach limits at $P_{CO_2} = 60$ mm. At these pressures of CO_2 the fraction of the oxygen capacity remaining in the blood is as follows: catfish 1.00; carp 0.90; yellow perch 0.77; sucker 0.58; chain pickerel 0.54; brook trout 0.50; rainbow trout 0.48; brown trout 0.50; lake trout 0.40. The species take the same order on the basis of either ease of saturation with oxygen or magnitude of CO_2 effect.

The effect of CO_2, which must facilitate unloading of oxygen into the tissues, should also hamper oxygenation if CO_2 is present in the water passing over the gills. The ability of the fish to utilize oxygen from water is cut down by CO_2 and in the same order for the species as the order of the CO_2 effect. Trout are unable to utilize oxygen in the presence of much CO_2, but catfish tolerate large pressures of CO_2. While the P_{CO_2} necessary to prevent completely the utilization of oxygen is larger than would occur naturally, the sensitivity of trout to CO_2 is such that some naturally occurring conditions would hamper oxygen utilization and hinder or prevent respiration in such water. In this manner it is shown that the specific properties of blood of fish which facilitate respiratory transport under some conditions would serve as definite barriers to existence of the fish under other conditions.

JULY 25

The relation between fermentation and respiration in higher plants (The Pasteur Effect).[1] David R. Goddard.

The experimental material was thin slices of cortical root tissue of the carrot (*Daucus carota*). All measurements of gaseous exchange were conducted in the Fenn micro-respirometer at 22° C.

It was found that carrot root respiration was strongly inhibited (75–85 per cent) by 10^{-3} M HCN or NaN_3 and about 65 per cent by 95 per cent CO. The CO inhibition was reversed by light. The partition coefficient of the oxidase for CO and O_2 was 9. The results are strong evidence that the major part of the respiration is catalyzed by cytochrome oxidase. Since these same poisons at the above concentration (and 100 per cent CO) did not inhibit fermentation in carrot, these poisons may be used to separate fermentation and respiration in the carrot under aerobic conditions.

Unpoisoned carrot tissue did not show any aerobic fermentation, but the anaerobic fermentation was high. These results demonstrate the existence of the Pasteur effect. As the O_2 pressure is lowered the rate of O_2 consumption begins to fall at about 5 per cent, but fermentation does not occur until $2\frac{1}{2}$ per cent or less. In fact, a very considerable inhibition of respiration (about 45 per cent) may be obtained before fermentation begins. With decreasing respiratory rate, the rate of fermentation increases. Aerobic fermentation may be obtained by poisoning respiration with HCN or NaN_3, but no fermentation occurs until the respiratory inhibition is 45 per cent or greater. In the presence of CO aerobic fermentation also occurs, and this effect may be overcome by light. These experiments indicate that the mechanism of oxygen inhibition of fermentation is through cytochrome oxidase. In the carrot root it has been impossible to poison the Pasteur effect, that is, to obtain aerobic fermentation without respiratory inhibition, with low O_2 tensions, HCN, NaN_3, or ethyl carbylamine. These results are all consistent with Meyerhof's oxidative resynthesis theory as an explanation of the Pasteur effect. They do not prove this theory. The above experiments are consistent with Lipmann's explanation only in the special case that the oxygen oxidation of the fermentation enzymes is catalyzed by cytochrome oxidase.

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[1] The results reported here are based on experiments of Mr. Paul Marsh and the author; and a full report will appear shortly in the *Amer. Jour. Bot.*

The rôle of bacteria in the fouling of submerged surfaces. Claude E. ZoBell.

Studies on the sequence of events in the fouling of submerged surfaces reveal that bacteria are the predominating primary film formers. Within an hour or two after the immersion of clean glass slides in the sea, bacteria begin to attach thereto. Some of the bacteria are so tenaciously attached that they resist dislodgement when the slides are washed under running water during the staining procedures, while others are only loosely associated with the primary film. The number of bacteria increases geometrically with time until their abundance together with the simultaneous and subsequent attachment of other organisms and detritus defeat census attempts. The adsorption and accumulation of organic matter on the submerged surfaces which has been demonstrated by chemical as well as by biological procedures is believed to account for the attachment and development of bacteria, although there are other ways in which solid surfaces promote the growth of bacteria in dilute nutrient solutions.

Barnacles, hydroids, bryozoa, tunicates, algae and other fouling organisms attach to submerged surfaces coated with films of bacteria more readily than to bacteria-free surfaces. Bacteria might promote the fouling of submerged surfaces (*a*) by affording the larval forms of larger fouling organisms a foothold or otherwise mechanically facilitating their attachment, (*b*) by serving as food, (*c*) by discoloring bright or glazed surfaces, (*d*) by increasing the alkalinity of the film-surface interface thereby favoring the deposition of calcareous cements, (*e*) by influencing the e.m.f. potential of the surface or (*f*) by increasing the concentration of plant nutrients at the expense of the organic matter which the bacteria decompose.

Cell division and differentiation in living plant meristems. E. W. Sinnott.

A paper containing the material given in this talk is to be published by E. W. Sinnott and Robert Bloch under the title: "Changes in intercellular relationships during the growth and differentiation of the living plant tissues," in the *Am. Jour. Botany* for October, 1939.

August 1

The ionic permeability of frog skin as determined with the aid of radioactive indicators. Leonard I. Katzin.

The rate of penetration of ions through the excised skin of the frog was measured using radioactive isotopes as markers for the salts which had passed through. Radioactive sodium (as the chloride) was used alone and in mixtures with inactive potassium chloride; radioactive rubidium, also in the form of the chloride, was used alone and in mixture with inactive sodium chloride. Diffusion was measured for two-hour periods between isotonic salt solutions bathing the inner side of the skin, and distilled water on the outside. The results are expressed in terms of the permeability to the pure salt in each case, and parentheses used to indicate extrapolated or estimated values.

Fraction NaCl	1.0	0.75	0.67	0.50	0.25	0.10	0.0
Permeability	1.0	1.0	1.1	1.15	1.6	1.8	(2.0)
Fraction RbCl	0.0	0.25	0.33	0.50	0.75	—	1.0
Permeability	(0.5)	(0.5)	(0.65)	0.66	0.7	—	1.0

The absolute rates of passage for the pure salts are 4.7×10^{-12} mols cm.$^{-2}$ sec.$^{-1}$ for sodium chloride and 125×10^{-12} mols cm.$^{-2}$ sec.$^{-1}$ for rubidium chloride. Values for the permeability ratio of RbCl and NaCl vary between 11 and 15, but are essentially constant. These results indicate that KCl (and probably RbCl) exert small influence on the permeability level of the frog skin, and that the passage of individual salts is essentially independent. Further, the large differences in rate of passage of rubidium and sodium ions indicates what has long been postulated, that the mobility of ions through tissues is not the same as in pure aqueous solution. An expression has been derived for the electrical resistance of the skin, from the Nernst diffusion coefficient and the Fick diffusion equation, on the assumption that the salts studied are the principal carriers of the electrical current. Sample calculations show again that there are differences in the ion mobility through the tissue.

Crystallization of myogen from skeletal muscle. Kenneth Bailey.

The main globulin component of skeletal muscle, myosin, is now well characterized. The separation of the remaining components, Weber's globulin X and the albumin myogen, has depended upon fractionation by dialysis, a procedure unsuited to many tissue proteins which denature in an ion-free solution. An attempt has been made to crystallize myogen by two methods: (1) The perfused, freshly excised minced muscle from rabbit is extracted with 10 per cent KCl at pH 7, and the solution after filtration through pulp is treated with ammonium sulphate which is dialyzed in through a collodian bag at room temperature. The precipitated myosin is filtered off when the specific gravity of the solution reaches 1.14. The solution is now brought to a pH of 6 by addition of sulphuric acid, and more ammonium sulphate is fed in until a specific gravity of 1.18 is attained; the amorphous globulin precipitates are separated, and the sulphate concentration again increased very slowly. Between a specific gravity of 1.18 and 1.22 the myogen separates in crystalline form together with some amorphous material which dissolves on cautious dilution, leaving the crystals in suspension. (2) The minced muscle is mixed with an equal volume of ice cold water and after standing for 20 minutes the juice is expressed. This is treated as outlined above.

The crystallization of myogen has recently been reported[1] by Baranowski; two crystal types were obtained, the one, termed myogen *A*, crystallizing as hexagonal bipyramids and the other, myogen *B*, as long thin plates. The *A* form is obtained by fractionation of muscle press juice by a procedure which involves a heat treatment at 50°, and the *B* form, which appears to be identical with the author's preparation, crystallizes fortuitously from the mother liquor. The physico-chemical properties of myogen *B* (purity by classical solubility methods, molecular weight, dielectric increment, titration curve) are now being investigated.

Chemical and histochemical observations on Macracanthorhynchus hirudinaceus. Theodor von Brand.

The females of *Macracanthorhynchus hirudinaceus* contain 1.13 per cent glycogen and 0.95 per cent ether extract. Phospholipids, unsaponifiable matter and unsaturated fatty acids are the chief components of the ether extract. Relatively more glycogen than fat is stored in the body-wall, whereas the contrary is true for the reproductive cells. Differential staining showed that the chief places of glycogen deposition are the hypodermis, the muscles and the mature eggs, those for the fat are the hypodermis and the ovaries.

pH reactions during feeding in the ciliate Bresslaua (Accompanied by photomicrographs taken on Kodachrome). C. Lloyd Claff and G. W. Kidder.

The large ciliate *Bresslaua*, a carnivorous member of the family Colpodidae, was used for experiments and observations on the pH condition during various phases of feeding and digestion. The general method was as follows: *Bresslaua* were treated with dilute indicator dyes and were later fed untreated *Colpoda steini*, the subsequent color changes studied under the water immersion lens. When neutral red- or methyl red-treated animals were used the color changes were very striking while other indicators gave less satisfactory results.

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[1] Baranowski, *Zeitschr. f. Physiol.*, 260, 43 (1939).

In neutral red preparations the medium, being slightly alkaline, shows a pale yellow coloration which extends into the oral cavity. As the first *Colpoda* is taken into the mouth a secondary vacuole forms in the posterior protoplasm to receive the prey. The contents of this vacuole appears faintly pink but as the prey is trapped by a thin sheet of protoplasm cutting the secondary vacuole off from the mouth region the periphery of the vacuole is outlined by a collection of brilliant red granules or droplets from the cytoplasm of the carnivore. The prey is immediately killed. Within a few seconds the granules disappear from view. The prey does not take on any of the red coloration at this time but slowly becomes yellow, then, as digestion proceeds, through orange to a cherry red.

The conclusions reached were that the actual killing of the prey is brought about by some strong acid. A pressure system was considered but ruled out by direct observation, in that the cilia of the prey stand out very straight from the body at the moment of killing. Methylene blue-treated *Colpoda* showed no color reduction, therefore lack of oxygen as a means of killing seems improbable. The color changes in the food masses subsequent to killing indicate that digestion is alkaline in nature and that the final residuum shifts back to an acid state.

August 8

The dielectric properties of insulin solutions. J. D. Ferry.

The material contained in this talk will appear shortly in *Science* under the title: "Studies in the physical chemistry of Insulin. I. The solubility and dielectric properties of insulin and its crystallization with radioactive zinc," by Edwin J. Cohn, John D. Ferry, J. J. Livingood, and Muriel H. Blanchard.

The effects of lack of oxygen, and of low oxygen tensions, on the activities of some Protozoa. J. A. Kitching.

A comparative investigation was undertaken of the effects of lack of oxygen, of low oxygen tensions, and of some respiratory narcotics, on the activity of Protozoa. The organisms were suspended in a thin hanging drop, and oxygen-free hydrogen or nitrogen (purified over hot platinized asbestos or hot copper), or hydrogen mixed with oxygen in known proportions by means of flow meters, were flushed continually *via* lead tubing with seals of de Khotinsky cement through the observation chamber. The purity of the oxygen-free gas was checked by the extinction of luminescence of marine luminous bacteria and by mass spectrographic analysis—methods sensitive to about 0.005 mm. of oxygen and to one part in 10^5 respectively.

In the peritrich ciliate *Cothurnia kellicottiana* pure hydrogen caused an almost immediate stoppage of the contractile vacuole and cilia, and a swelling of the body; very often the body blistered. Return to air led to an immediate recovery of the vacuole to a rate of output at first much above the normal. The cilia resumed their beat, and the body slowly shrank to its original size or less. A partial pressure of 1.1 mm. of oxygen was not enough to allow any vacuolar activity; at 1.6 mm. the contractile vacuole stopped but recovered slightly after the body had swollen; and in 3 mm. there was full activity. In dilute cyanide there was little or no activity of the contractile vacuole.

In fresh-water amoebae of the "proteus" type the contractile vacuole quickly ceased all activity in absence of oxygen, or in cyanide; although amoeboid movement continued for some hours. Recovery in air from lack of oxygen was rapid. Amoeboid movement and the activity of the digestive vacuoles of the marine amoeba *Flabellula* *mira* were rapidly and reversibly inhibited in absence of oxygen. Some measure of recovery from lack of oxygen was obtained at 0.3 mm.

Paramecium spp. continued swimming for some time in the absence of oxygen, but eventually stopped and cytolysed. The best survival, namely 12 hours, was obtained in culture medium and with sufficient carbon dioxide added to the hydrogen or nitrogen to maintain the pH at a reasonable value. Anaerobic survival was much shorter in dilute phosphate buffer, or for starved animals. Some recovery from lack of oxygen was obtained in 0.3 mm. oxygen.

No correlation could be found between the degree of sensitivity of Protozoa to cyanide and the critical oxygen tensions at which these organisms were just able to maintain some activity.

Nerve asphyxiation and aerobic recovery in relation to temperature.[1] Herbert Shapiro.

The sciatic nerve of the Hungarian bullfrog, *R. esculenta*, was mounted on 3 platinum electrodes for stimulating, and 2 calomel recording electrodes in an all glass chamber, which could be immersed in a Dewar flask and thus kept at constant temperature over long periods. Moistened purified nitrogen or hydrogen was passed through the chamber, and test stimuli from a commutator permitting condenser charges and discharges through the nerve at any desired frequency, were applied at regular intervals. The duration of the tetanus was controlled with a Lucas contact breaker. The total action current was integrated ballistically by a Zernicke Zd galvanometer of 4-second period. During anaerobiosis, the response of the nerve to a standard stimulus gradually decreases, and finally is extinguished. At higher temperatures "overshoot" occurs during early anoxia. After positivity disappears earlier than action current, but injury potential was never completely abolished during the experiment. Continuous tetanization shortens asphyxiation time. Time for asphyxiation of action current is an exponential function of temperature, requiring approximately an hour at 38° C., and about 1150 minutes at 0° C. The form of the function may be described by the Arrhenius equation, yielding a temperature characteristic of 11,100 calories. Evidently the nerve utilizes energy from an anaerobic reaction for setting up and conducting action currents, and this reaction runs to completion at a rate determined by the temperature. Upon readmission of oxygen to the nerve, action current, injury potential and after positivity showed recovery. Rate of recovery of action current also conformed to the Arrhenius equation with a μ value of 28,000 calories. Examination of Amberson's data for temperature effect on the absolute refractory period of nerve shows a μ value of 18,400. Underlying chemical reactions for conduction, refractory period, and recovery in nerve proposed by Gerard are such as to suggest three different types of reaction, and hence different temperature characteristics. Though the latter by themselves do not identify the reactants involved, it is of interest that the present study indicates chemical bases for these several aspects of nerve activity, with different enzyme systems acting as the controlling links.

Effects of hydrostatic pressure upon certain cellular processes. D. A. Marsland.

The experiments demonstrate that pressure induces a solation of protoplasmic gels in a number of different cells and in an inanimate gel prepared of the myosin of rabbit muscle. In each of the cells studied, the solation is associated with a retardation of movement—amoeboid movement, the pinching of the cleavage furrow, and the streaming of plant cells.

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[1] This work was done during the tenure of a fellowship of the John Simon Guggenheim Memorial Foundation.

In order to see cells while under compression, it was necessary to construct a special chamber. This chamber permits objects within it to be viewed at a magnification of 600 diameters at pressures up to 600 atmospheres.

The pressure effects upon the rigidity of the cellular gels were measured by the centrifugal method. A centrifuge head was constructed which permits centrifugation during the period in which the cells are under compression.

When amoebae are suddenly exposed to a relatively high pressure (above 450 atmospheres) a peculiar reaction suddenly occurs. Each of the extended pseudopodia collapses and tends to round up. This result would be expected if the plasmagel, which supports the pseudopodia, were to undergo liquefaction. If the pressure is maintained for a few minutes, the whole cell becomes spherical. However, when the pressure is released, active amoeboid movement begins again within a minute or so.

Centrifuge experiments demonstrate that the form of the amoeba is related to the structural properties of the plasmagel. When the amoebae are centrifuged under pressure, the liquefaction is indicated by the rapidity with which the granular components of the plasmagel undergo displacement. In the higher range of pressure, the rigidity of the plasma gel is reduced to a small fraction of the normal value. In this range of pressure, no pseudopodia may be sustained. In the lower range, pseudopodia may be formed, but they display a graded diminution of diameter and length as the pressure is increased.

Comparable results were obtained in studies of cleaving *Arbacia* eggs and the streaming of the leaf cells of *Elodea*. The retardation of the furrowing parallels the loss of rigidity of the gelated cortex of the egg, and the streaming of the *Elodea* cell is slowed in proportion to the degree of liquefaction which occurs in the non-flowing part of the protoplasm. Thus it appears that sol-gel reactions are providing a machine by which the cell can transform chemical potential energy into mechanical work.

August 15

On the nature of the material elaborated by fertilizable Nereis eggs inducing spawning of the male. Grace Townsend.

The observations of Lillie on the spawning reaction of *Nereis* limbata led to the formation of the "fertilizin theory" (Just, 1930). Lillie found evidence that the material inducing spawning of the male came only from fertilizable eggs.

I have re-investigated the relation of the spawning inducing material to fertilization and have found it to possess properties in common with material essential to egg activation though not necessarily associated with the phenomenon of egg and sperm union.

Egg-cell activation may plausibly involve processes common to all species and be based on the same processes as may initiate cell division in any tissue. Correspondingly, spawning was induced by extracts of many fresh tissues from many species, all of which contain glutathione. Crystalline pure glutathione in one part in a million in a single drop quantity, and the molecular constituent, cystine or cysteine, in higher concentration, induced the natural spawning reaction.

Marine eggs are rich in glutathione (300–700 mg. per 100 gm. wet weight) and it is especially concentrated in the germinal vesicle. A reducing substance passes from *Nereis* eggs. The reaction of the male to glutathione, by a large variety of tests, was indicated to be extremely specific.

All substances found to destroy the spawning inducing property of egg-water and glutathione inhibit fertilization.

Properties of Egg-Water Spawning Inducing Material and Glutathione and "Fertilizin" and Its Described Fractions

Analysis of properties:	Egg-Water Spawning-Inducing Material	Glutathione	Woodward's		Lillie's Fertilizin
			Parthenogenetic Fraction	Agglutinin Fraction	
Elaboration from egg	+	+	+		+
Xanthoproteic test	−	−	−		−
Stability in acid	+	+			
Stability in alkali	−	−			
Precipitation by acetone	+	+	+		
Berkfeld filter	+	+	+	−	−
Dialysis	+	+	+	−	−
Inactivation by aging	+++	+	+++	+	+
Inactivation by boiling in s. H_2O	+++	+	+++	+	+
Inactivation by irradiation	+++	+	+++	+	+
Inactivation by Au, Cu, Ni, etc.	+++	+	+	−	+
Inactivation by CH_2ICOOH	+++	+			
Inactivation by KCN	−	−			−
Inactivation by blood, coelomic fluid	+	+	+		+
Inactivation by cytolyzed eggs	+	+			+
Inactivation by cytolyzed tissues	+	+			
Induction of spawning	+	+			+

Ovum and spermatozoön age at the time of fertilization and the course of gestation and development in the guinea pig. W. C. Young.

An abstract of this paper has already been published in the *Anat. Rec.* by Arnold F. Soderwall, William C. Young and Richard J. Blandau, under the title: "Spermatozoa vitality in the genital tract of the guinea pig" (*Anat. Rec.*, vol. 73, Suppl. 2, p. 47, 1939).

Experiments on the production of haploid salamanders. Cornelius T. Kaylor.

The eggs of many species of amphibians can be induced to begin their development with only one set of chromosomes. This would then produce haploid embryos and larvae.

In spite of the large number of experiments which have been performed on the production of haploid amphibians (see review of Fankhauser, *Jour. Hered.*, 28, 1937), the results have varied with the species used, the degree of abnormality of embryos produced, and with the extent of development. So far as demonstrated, only one completely haploid amphibian has been reared to a stage approaching sexual maturity (Baltzer and Fankhauser, 1922). This is in striking contrast to the fact that normal haploid animals exist in nature, as well as to the fact that viable haploid plants have been produced experimentally.

The present experiments were undertaken primarily to test, by new methods, with the eggs of species which have not been used extensively before, the possibilities of advanced haploid development in these species. Two species of newts were used: *Triturus viridescens* and *Triturus pyrrhogaster.* The female chromosomes were removed from the egg with a small pipette. All subsequent development then took place by means of the male chromosomes.

In over 200 experiments with the eggs of *T. viridescens*, only about 15 per cent of the embryos developed beyond the gastrula stage. In 76 experiments with pyrrhogaster eggs, about 30 per cent of all embryos developed to stages ranging from a neurula to a 120-day-old larva. This larva, however, was not completely haploid. Another larva, which in external appearance resembles all haploid larvae which have been produced in other experiments, was fixed at 47 days of age, at a time when hind limb buds had appeared. It had a deformed jaw and could not take food.

It appears, then, that for the purpose of obtaining advanced haploid larvae in these amphibians, the eggs of *Triturus pyrrhogaster* are much more adaptable than eggs of *Triturus viridescens*.

Regulation in mosaic eggs. Alex B. Novikoff.

Transplantation experiments on the eggs of *Sabellaria vulgaris* by the author[1] have demonstrated that the materials in the polar lobe which are involved in the formation of the apical tuft and the post-trochal region do not diffuse from the transplanted lobe into adjacent cells. But there were some indications that when the polar lobe material was incorporated into a cell the course of development of the cell was altered.

A 7.5 per cent solution of 2.5 Normal KCL in sea water inhibits cleavage in the eggs of *Sabellaria*. Eggs are placed into the solution after the second maturation division, and kept there until the controls have completed the first cleavage. Upon returning them to sea water, as many as 90 per cent of the eggs develop into perfect double embryos. These embryos possess two eye spots, two sets of posttrochal bristles, two posterior cilia, two sets of dorsal cilia, two neurotrochs, two intestines, one central stomach, probably one oesophagus, one mouth, and two mouth folds.

At least some of the cells in these larvae have developed into structures which they do not form in normal development. Thus the prospective potency of these cells is revealed to be wider than the prospective fate. This is a characteristic generally associated with regulative eggs.

Eggs placed in the KCL solution *after* the completion of the first cleavage until the controls have completed the second cleavage do not develop into double embryos. Instead larvae which possess extra bristles, or extra eye spots, or both, are produced.

The formation of double embryos suggests that the material of the first polar lobe has been distributed to each of the first two blastomeres where it 'organizes' in each a new embryonic axis.

August 21

Micromanipulative studies. (*Motion picture*). Robert Chambers.

Living cells in action (*motion pictures*). C. C. Speidel.

Ciné-photomicrographs of the fast motion type have been taken of many kinds of cells. The pictures are made directly from living frog tadpoles and they reveal characteristic cellular movements and reactions under normal and experimental conditions.

The pictures include examples of the growth, migration, mitosis, and differentiation of connective tissue cells, epithelial cells, vacuolated sub-epidermal cells, endothelial cells of blood and lymph capillaries, sheath cells, regenerating spinal cord cells, pigment cells, and various kinds of leucocytes. A complete record of nerve regeneration over a period of a month is given, including the stages featured by growth cones, sheath cells, and myelin segments.

Case histories are also presented to show the changes in position from day to day of the relatively stable cutaneous nerve endings which belong to myelinated fibers. These include examples of extension, retraction, irritation, autotomy, and new growth cone differentiation following loss by phagocytosis. Several cases are given which reveal how red blood cells that have been extruded from blood vessels are engulfed by macrophages.

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[1] *Biol. Bull.*, 1938, **74**, p. 211.

Various types of behavior of localized contraction nodes in single muscle fibers (from *Palaemonetes* leg and *Limulus* heart) are also presented. These include their formation, progression, splitting, reflection, collision, and dissipation; also their progression past thin clots resembling intercalated discs.

Other pictures (obtained with the coöperation of Dr. Ethel Harvey) show the early developmental history of the sea urchin, *Arbacia*, including the immature egg, mature egg just before and just after fertilization, segmentation stages from 1 to 64 cells, free-swimming gastrula, and pluteus. Other pictures show abnormal cleavages of centrifuged eggs and of the clear halves of centrifuged eggs.

Polariscopic pictures reveal the birefringent substances in pigment cells, epithelial cells, muscle fibers during contraction and relaxation, and in the developing eggs and larvae of *Arbacia*.

August 22

Studies on the life history of Spelotrema Nicolli. R. M. Cable and A. V. Hunninen.

Metacercariae occurring in the blue crab, *Callinectes sapidus*, were fed to six young herring gulls which were killed and examined 12 hours to 18 days later. Each bird yielded a large number of adult *Spelotrema nicolli* while six controls were negative for this species. Since very young metacercariae indicated clearly that the cercaria is of the Ubiquita type, an extensive search was made for such a larva in mollusks occurring where infected crabs were abundant. After many unsuccessful attempts, a cercaria of the type sought was found in the snail, *Bittium alternatum*. This cercaria was found to enter the crab by way of the gills, passing with the circulation to strands of muscle-like tissue where encystment occurred. The morphology of various stages in the life history of *S. nicolli* has been studied in detail; the excretory formula of the cercaria is $2[(1 + 1) + (1 + 1)]$, the metacercaria and adult, $2[(2 + 2) + (2 + 2)]$.

Stabilizing action of alkaline earths upon crab nerve membranes, as manifested in resting potential measurements. Rita Guttman.

Resting potentials of the non-myelinated nerve of the proximal segment of the claw or of the first walking leg of the spider crab, *Libinia canaliculata*, were measured by means of a potentiometer and null point galvanometer. All solutions used were approximately isotonic with sea water and pH was controlled.

It was found that the alkaline earths, in themselves, have no effect upon the magnitude of the potential. Yet they are able to prevent the usual depressing action of KCl upon the potential when the alkaline earth and KCl are simultaneously applied to the nerve. Solutions containing two parts of $BaCl_2$ to one part of KCl, five parts of $SrCl_2$ to one part of KCl, and eleven parts of $CaCl_2$ to one part of KCl are threshold values for the neutralization effect. The order of effectiveness of the alkaline earths for counteracting the depression of the potential by KCl is thus Ba, Sr, Ca. This is the order in which these elements appear in the atomic table.

The alkaline earth, Ba, is also capable of preventing the depression of the resting potential by various lipoid-soluble, surface active, highly polar substances, viz., veratrine sulphate, chloral hydrate, iso amyl urethane, sodium salicylate and saponin.

The neutralization effect of the alkaline earths may last over a period of many hours. The alkaline earths are capable of preventing the action of depressants strong enough to cause, when present alone, a decrease of potential of fifty per cent or more.

A direct relationship was found between the amount of depression of the potential per unit time caused by veratrine sulphate and the logarithm of the concentration of veratrine sulphate acting. Although a concentration effect is obtained with both K and veratrine sulphate, it is not necessary to assume that the action of these substances upon the nerve is identical. It should be noted that the relative concentration of KCl (one part isotonic KCl to eleven parts sea water, or 0.04 M) necessary to depress the potential is of a much greater order of magnitude than that of the organic substances (0.00004 M veratrine sulphate in sea water).

Two possible explanations for the neutralizing action of the alkaline earths are: (1) change of effective pore size in a sieve-like membrane by the alkaline earths or (2) alteration of the partition coefficients of the depressants by the alkaline earths. There is at present no basis for deciding between these alternative concepts. The phenomenon is certainly, however, not one of antagonism in the classical sense of Jacques Loeb, inasmuch as, for one thing, the quantities of alkaline earth necessary are much too large.

An experimental study of the pigment granules of the Arbacia egg. D. L. Harris.

The pigment granules are isolated by breaking eggs in 0.35 m Na_3 citrate to avoid the presence of Ca. Unbroken cells are removed by centrifuging. It is found that the granules are actually vacuoles. They are easily deformed, and regain spherical shape when external force is removed. In the presence of Ca, Mg, and Sr; hypotonic solutions; and isotonic solutions of urea, acetamide, and ethylene glycol they discharge pigment. Moreover, in Ca, Mg, and Sr solutions, contiguous granules coalesce to form fluid vacuoles containing small granules in active Brownian movement.

The discharge of pigment in hypotonic solutions was investigated quantitatively. The number of granules present at various concentrations of Na_3 citrate was determined by counting. It was found that there was much more extensive breakdown at low concentrations. Furthermore, there is a normal distribution of granules in respect to ease of breakdown.

In the absence of Ca, Mg, or Sr, the pigment outside of the granule turns greenish brown and finally green. A suspension of granules in Na_3 citrate treated with hypotonic solutions will therefore change color from pink to green. The amount of granule breakdown may be determined by comparing the color in a given hypotonic suspension with standards prepared by making mixtures of pink suspension and green suspension. This method gives results in agreement with those of the counting method.

The rate of granule breakdown in various hypotonic concentrations was determined by measuring the time to reach a given color, *i.e.*, a given amount of breakdown. Using eight end-points, it was found that as the concentration was decreased the rate of reaction increased. This is clear evidence that the granules behave as osmometers. It is concluded that the granules are vacuoles surrounded by a semi-permeable membrane, and that they do not constitute osmotic dead space within the cell.

The action of calcium on muscle protoplasm. L. V. Heilbrunn.

The complete paper is in press and will appear shortly in *Physiol. Zool.*

GENERAL SCIENTIFIC MEETINGS

AUGUST 29

The mechanism of membrane elevation in the egg of Nereis. D. P. Costello and R. A. Young.

Certain agents may induce the formation of a very wide perivitelline space in the *Nereis* egg. Alkaline NaCl (pH 10.5) was used by D. P. Costello, and X-rays (8,800–41,300 r) were used independently by R. A. Young.

When placed in alkaline NaCl the uninseminated egg elevates the vitelline membrane (occasionally double), and the width of the perivitelline space increases until the membrane ruptures, setting free the ovum. It was demonstrated by means of a Chinese ink suspension and by microneedles, that there is no jelly external to the intact membrane, but that jelly fills the perivitelline space and escapes with the ovum upon membrane rupture. Attempts to inseminate such eggs after return to sea water have been unsuccessful. Alkaline NaCl is a parthenogenetic activating agent. If normally inseminated eggs are subsequently treated with alkaline NaCl, the passage of cortical jelly through the membrane ceases, and a perivitelline space appears of width inversely proportional to the jelly already extruded. Protoplasmic cone-shaped filaments, temporarily adhering to the elevating membrane, are gradually retracted, except the one to which the activating spermatozoön is attached. The spermatozoön passes through the membrane 7–10 minutes after the egg is introduced into alkaline NaCl. The penetration of the sperm into the treated egg takes place three times as rapidly as under normal conditions, and can be followed with unprecedented clarity.

If *Nereis* eggs are treated with X-rays, and inseminated after 5–10 minutes, a similar, but much slower, response is induced. The width of the perivitelline space is directly proportional to the dosage, whereas the extrusion of jelly through the membrane is inversely proportional to dosage. In these eggs the sperm frequently does not complete the penetration process.

The colloidal nature of the perivitelline material is demonstrated by collapsing the elevated membrane against the egg surface with a solution of gum arabic in sea water.

It is suggested that exaggerated membrane elevation of the *Nereis* egg may be obtained by agents which (1) initiate the outflow of the cortical jelly precursor (if the egg has not been previously inseminated), and which (2) alter either the vitelline membrane or the jelly in such a way that passage of the jelly through the membrane is completely or partially prevented.

Determination and induction of the anuran olfactory organ. Edgar Zwilling.

By means of large transplants of presumptive head ectoderm from gastrulae of varied ages to the flank of older urodele embryos it was established that the olfactory organ of *Rana pipiens* is determined (*i.e.* would self-differentiate) before the neural folds are present. This determination probably occurs in the small yolk-plug stage. The presumptive olfactory material of pre-neurula stages and of early neural plate stages self-differentiated in the absence of brain tissue. Since it was possible that the presumptive brain tissue might act laterally upon cells in the same layer and so be responsible for the determination, the brain tissue was tested for its inductive capacity. Forebrain material from various stages during neurulation was transplanted beneath the epidermis of the flank region of various host stages. In only one case did olfactory material develop; and since this operation was an heteroplastic one (to *Rana palustris*) it could be determined that the olfactory tissue was of donor origin. The anterior portion of the roof of the archenteron was then implanted into the blastocoeles of older gastrulae to test its inducing power for this structure. Of 19 surviving cases 5 developed small but perfect heads—which had olfactory organs. One of the others developed an olfactory organ in the absence of brain. Of 9 controls (where forebrain of the open neural plate stage was implanted into similar gastrulae) one developed an olfactory canal. The origin of this tissue cannot be established with certainty since this was an homoplastic graft.

A method of determining the sex of Arbacia, and a new method of producing twins, triplets and quadruplets. Ethel Browne Harvey.

To determine the sex of *Arbacia*, inject a few drops of a saturated solution of NaCl in sea water into the mouth; then with a fine pipette inject a little of the same solution into *one* genital pore. A few eggs or a little sperm will exude *only* from the opening injected, easily distinguishable by color. If thrown immediately into a jar of still sea water, the animal will stop shedding, and the gonads remain normal and intact. If the animal does not respond to NaCl, a molar solution of KCl can be used; this is more drastic.

To obtain twins, place the eggs from which fertilization membranes have been removed in a solution of 30 grams of NaCl per liter of sea water for 5 to 10 minutes, immediately after first cleavage; then remove to sea water. The first two blastomeres are nicely separated, but connected by a thin strand, and they develop independently. The blastulae at first swim in pairs and can easily be isolated. Some pairs gave both normal plutei, some both abnormal, and some, one normal one abnormal. In lots of 10 pairs, development was variable, but in one lot all but one became perfect or almost perfect plutei. Quadruplets are obtained by similar treatment just after second cleavage; four almost perfect plutei have developed from the first four blastomeres in some cases. Triplets are obtained when two of the blastomeres of the 4-cell stage develop independently, and the other two together.

An artificial nucleus in a non-nucleate half-egg. Ethel Browne Harvey.

The non-nucleate half-eggs of *Arbacia punctulata*, obtained by centrifugal force, have been injected (by W. R. Duryee) with yeast nucleic acid and thymus nucleic acid. The injected drop of fluid does not disperse through the cytoplasm, as does sea water, but remains distinct in a small drop or vacuole surrounded by a film or membrane, resembling strikingly the appearance of the nucleus in the living cell. The possibility of supplying the non-nucleate eggs with chemical materials found in the normal nucleus opens a new line of investigation in the effort to cause the parthenogenetic merogones (*i.e.* activated non-nucleate egg fractions) to develop beyond the blastula. Nucleic acid, nucleo-proteins, adenine, guanine, uracil, auxins, tobacco mozaic virus and many other substances, added to the medium before, during and after centrifugation have been found to have no effect.

Color responses of catfishes with single eyes. G. H. Parker.

As a rule fishes with only one eye respond by color changes to differences in their environment as successfully as do those with two eyes. In this respect the trout has long been known to be peculiar for on the loss of one eye it darkens contralaterally. The common catfish, *Ameiurus nebulosus*, when deprived of one eye conforms neither to the general rule for fishes nor to the special one for the trout. A one-eyed catfish is at first very dark, after which it may change slowly in tint according to its environment though without ever becoming fully pale. Such fishes may finally assume in the same environment somewhat different tints and retain these with considerable individual persistence. This diversity appears not to be due to variations in the irritability of the orbital wound which might influence to various degrees the stump of the optic nerve. The cause of these more or less characteristic color differences is unknown. In these respects one-eyed catfishes are unlike any other fishes thus far described. The fact that intermedin, the secretion from the pituitary gland, plays a very important part in the color changes in catfishes, and that in this fish chromatophoral nerves, both dispersing and concentrating, are less significant than the pituitary gland, may be the occasion of the difference between *Ameiurus* and most other teleosts whose chromatophores are often under almost exclusively nervous control.

A vibration sense in a swarming annelid. Grace Townsend.

During sexual metamorphosis of *Nereis limbata* a vibration sense becomes highly developed which equips the worms for orientation in rapid swimming. The sense enables the worms, typically, to avoid striking solid objects, to relate themselves to smooth surfaces and to the opposite sex. Circling can be induced by the vibration sense alone and the sense doubtless supplements the chemical sense (Lillie and Just, 1913) in the spawning integration of this species. The marked development of the sense is associated with the metamorphosis of the dorsal and ventral cirri.

During metamorphosis the cirri become elongated beyond the surrounding parapodial structures and equipped with budding elevations, or processes, along the inner surfaces bordering the parapodial lobes. The elevations are filled with nerve cells which connect with clusters of turgid cytoplasmic hairs.

If the cirri are clipped from the parapodia of the metamorphosed worms (with use of iridectomy scissors), the worms lose their characteristic orientation to surfaces and swim unrelated to surfaces as does the non-sexual phase.

The sensory structures of the dorsal and ventral cirri are part of the lateral line system of annelids elaborately described for the Capitellidae by Eisig (1879) and for all groups by Jeener (1928). Treadwell (1900) describes a striking sensory organ on the dorsal cirrus of a swarming palolo (*Eunice auriculata*). No work has been done as to the general functional significance of the system. Stolte (1932) observed that the dorsal cirri of *[illegible]era* are not stimulated by chemicals or tactile stimuli but are stimulated by slight mechanical oscillations of the structures.

The plausibility of a generally distributed vibration sense in annelids possessing a pelagic phase is suggested.

Food habits of Endomoeba muris. D. H. Wenrich.

These studies were made on prepared smears of the caecal contents of rats and mice, mostly fixed with Schaudinn's fluid and stained with iron alum haematoxylin.

Endamoeba muris feeds on a great variety of food materials including various kinds of bacteria, *Blastocystis*, yeasts, filamentous organisms, starch grains, *Trichomonas*, *Giardia*, *Hexamitus*, leukocytes and erythrocytes. On the same slide some individuals may be engorged with one kind of food while others will be engorged with different kinds; still others may contain a variety of food materials. There is a tendency for the amoebae from one particular host to show food preferences different from those from some other host. For example, on one set of slides about 60 per cent of the amoebae contained a certain kind of colonial bacteria, while on another set of slides about 75 per cent showed one or more individuals of *Trichomonas muris* in their food vacuoles.

In ingesting different kinds of food objects, *E. muris* employs somewhat different methods. Fairly large starch grains are apparently surrounded by slowly advancing pseudopodia on all sides in close contact with the grain, but one pseudopodium may be further advanced than the others. The edges of the advancing pseudopodia often stain intensely with iron alum haematoxylin. Some food cups were surrounded by a dense layer of cytoplasm which sometimes did and sometimes did not stain intensely. Ingestion cones were extended along plant filaments, which became coiled inside the cell. In most cases an ingestion cone included a definite pharynx-like structure with deeply-stained walls and annular thickenings. Similar pharynx-like tubes formed without protruding cones were employed in ingesting *Trichomonas* *muris.* Comparable tubes were also found deep in the cytoplasm apparently being used to break up food masses into smaller units.

A quantitative study of the hemopoietic organs of young albino rats. J. E. Kindred.

A study has been made of the differential cell counts and mitosis rates in the parotid lymph nodes, spleen, thymus and bone marrow of 15-day-old and 20-day-old albino rats. These data have been used in conjunction with differential cell counts of the blood and with the volumes of the several organs in an attempt to find out what degree of relationship exists between cell production and differentiation in the organs and the blood. Other investigators have suggested that in order to meet the demands of granulocytopoiesis and erythrocytopoiesis, the bone marrow must filter out small lymphocytes from the blood stream. The present data show that the lymph nodes and spleen are deficient in the production of small lymphocytes needed for the growth of these organs between the 15- and 20-day stages. On the other hand, the bone marrow and thymus produce an excess of small lymphocytes during this period. The excess small lymphocytes more than balance the needs of the lymph nodes, spleen and blood. The principal reason for the excess production of small lymphocytes (hemocytoblasts) in the bone marrow is an actual decrease in numbers with time, whereas in the thymus, the excess is caused by a very high mitosis rate in the peripheral zone of the cortex.

On the histology of the mammalian carotid sinus. William H. F. Addison.

The carotid sinus is the dilated beginning of the internal carotid artery at the bifurcation of the common carotid artery into the internal and external carotid arteries. The wall of the carotid sinus has the three layers characteristic of arteries, but its structure differs from that of adjoining regions of the arteries with which it is continuous by the presence in the tunica media of a predominant amount of elastic tissue and by the absence or the small amount of smooth muscle. In the sinus of the dog the elastic tissue is arranged in 8–10 coarse lamellae with collagenous tissue interspersed. The transition in structure from the sinus to the internal carotid artery is abrupt and there is a conspicuous difference in the organization of the walls. The diameter of the sinus is over twice as great as that of the adjoining internal carotid artery in the living animal. Ordinarily the carotid sinus may pass unnoticed in the dead animal because the lowering of blood pressure at death allows the sinus to diminish in size until it is only slightly larger than the diameter of the internal carotid artery. A type of carotid sinus similar to that in the dog has been seen in other mammals, *e.g.*, newborn child, rhesus monkey and cat.

On Clark's theory of muscular contraction. Alexander Sandow.

The theory of contraction for striated muscle proposed by Clark (*Am. J. Physiol.*, V. 82, p. 181, 1927) depends on the following assumptions: (1) the muscle fibers consist of alternately arranged isotropic and anisotropic layers which run unbroken across each fiber; (2) upon stimulation chemical changes occur which transform the liquid crystals of the anisotropic discs into a more solid crystalline state, while the substance of the isotropic layers remains unaltered; (3) the new relation between the substances of the layers results in the sudden production of a tension that can be calculated by means of the formula $F = 2AT/d$ where $F =$ the force per fiber, $A =$ the area of the fiber cross section, $T =$ the surface tension of the material of the isotropic layers, and $d =$ the thickness of this layer. Using the values for the frog sartorius: diameter of fiber $= 50\,\mu$, $T = 70$ dynes/cm., $d = 0.7\,\mu$, Clark finds $F = 39.25$ dynes, in fair agreement with the experimental value for maximal isometric tension of 55 dynes (Hill, 1926). Consideration of more recent work (Hill, 1938) and allowance for Fenn's chloride space lead to the value for F of 37.5 dynes, in striking agreement with Clark's calculated value. Although muscle physiologists have paid little attention to this theory, it has received some notice in physiological literature. (Evans, 1931; von Muralt and Edsall, 1930; Burns, 1929; Howell, 1936; Barnes, 1937.)

Clark's theory may be questioned on general grounds: the assumed value of T is probably too high; there is a possibility of contradiction between the predicted and observed changes in birefringence during contraction; the assumed mechanism for tension production is itself open to criticism; and even if it be accepted it cannot be applied to unstriated muscle. The theory is definitely at variance with observation in indicating that during contraction the isotropic bands shorten relative to the anisotropic bands. But difficulties of a decisive nature arise if, conforming to the generally accepted view (Schmidt, 1926; Hürthle, 1931; Chambers and Hale, 1932; von Muralt, 1933) account is taken of the fibrillar structure of the muscle fiber. Clark's method for calculating the tension must then be applied to the individual fibrils, and the sum of their tensions taken as the tension developed by the whole fiber. Now, however, the previously used formula is not valid, since the diameter of the fibril is of the same order as the thickness of the isotropic segment of a sarcomere. The correct formula for the tension per fiber is $F = 2nAT(1/d - 1/D)$, where n = the number of fibrils per fiber, A = the cross-sectional area of a fibril, d = the thickness of the isotropic segment, D = the diameter of the fibril, and T is as before. Using Clark's values for T, d, and the diameter of the fiber, and taking $D = 1\ \mu$ and n the value for closest packing of the cylindrical fibrils, it is found that $F = 10.6$ dynes. This is clearly far too low in comparison with the experimental value to support the theory. Moreover, the assumption that $d = 0.7\ \mu$ is open to question. The work of Buchthal, Knappeis and Lindhard (1936) has shown that d in the frog sartorius may vary from 0.81 μ to 1.10 μ depending on the degree of stretch of the muscle. If $d = 0.9\ \mu$, then $F = 2.8$ dynes. And if $d > 1\ \mu$, or if in general $d > D$, then F becomes negative indicating instability of the system and the separation of the fibrils into discrete sarcomeres. In view of the difficulties and inadmissable implications of Clark's theory we must conclude that it cannot be accepted as a valid picture of the mechanism of contraction of striated muscle.

Conditions governing the frequency of contraction of the heart of Venus mercenaria. Albert E. Navez and John D. Crawford.

Two characteristics: frequency and amplitude of the beat of the excised and perfused ventricular portion of the heart have been studied. This note is concerned only with the frequency.

If one plots against time, the frequency of the heart perfused with non-aerated sea-water, one obtains a curve showing a rapid rise (in about 2 hours) to a maximum frequency followed by a slow decline to zero in about 36 hours. Perfusion with aerated sea-water determines a frequency curve which reaches a plateau, slightly below the maximum value and which extends over 36 or more hours before final decline sets in. If a solution of one part of dextrose into 250,000 parts of well aerated sea-water is used as perfusion liquid, the plateau of constant frequency may be extended to 72 to 96 hours. The rate of perfusion is sufficient when above 20 ml./min.

Frequency is a function of load (tension) on the heart. The load determines the time elapsing between excision and maximum in frequency curve. In general less time is required to reach maximum frequency under greater loads. The graph of "time of excision to time of maximum frequency" against "load" is a hyperbola with a short induction period for loads below 30 mg. The "excess frequency" (frequency above that of the level plateau mentioned above) is also a function of the load. The value of the "excess" frequency is greatest at about 80–95 mg. load and is of smaller value for loads above or below this point.

For a heart beating at constant frequency under a given load, any change in load below a critical tension of about 200 mg. effects a change in frequency that is completely reversible. When the load on a heart is changed, the frequency changes correspondingly, reaching a new value through a series of "damped oscillations" over a period of more than an hour at room temperature. Above the critical load, changes in frequency are not exactly reversible. On passing from 100 mg. to 250 mg., the frequency rises; on return to 100 mg. the frequency falls decidedly below its original value.

In all experiments reported the temperature was kept constant to within ± 0.01° C.

Small variations in temperature affect decidedly the rate of contraction. Four (or five) temperature (characteristics) have been found for a large series of hearts studied. Critical temperatures have been found around 10°, 15°, 20–21°, and 25°; not all appear in every heart studied.

The refractory period in the non-conducted response of striated muscle. F. J. M. Sichel.

In the isolated skeletal muscle fibre (frog), with cut ends, the contractile mechanism can be excited to a normal type of response apparently without involving the conductile mechanism (Brown & Sichel, *J. C. C. P.*, **8**, 315, 1936; Sichel & Prosser, *Biol. Bull.*, **73**, 293, 1937). This non-propagated response has no absolute refractory period and the size of the response is a function of the strength of the stimulus even though the entire length of the fibre is involved in the contraction.

During the course of an extension of these experiments in collaboration with D. E. S. Brown use was made of the fact that twitch-like contractions without propagation can also be obtained in the intact entire sartorius provided the KCl content of the medium is 70 mg. per cent. If the entire muscle is stimulated under these conditions by means of massive electrodes so placed that the electrical field is at right angles to the longitudinal axis of the muscle, the entire muscle is involved in the contraction. Recorded isometrically these contractions have a normal form. They resemble the contractions of the isolated fibre preparation and differ from those of the normal muscle in that they are essentially local and non-propagated and also in that no refractory period is involved in their excitation. The absence of the refractory period was demonstrated as in the case of the isolated fibre preparation by stimulating with two equal condenser discharges separated by a variable time interval. For the whole muscle stimuli of rectangular form were also used. In the isolated fibre and in the KCl-treated muscle the second stimulus will always contribute something to the mechanical response; in the normal muscle there is an interval, related to the absolute refractory period, during which the second stimulus can contribute nothing. Since the response of the KCl-treated muscle is non-propagated, its grading presumably does not necessarily involve the frequency distribution of the fibre thresholds.

Pigment inheritance in the Fundulus-Scomber hybrid. Alice M. Russell.

Hybridizations between *Fundulus heteroclitus* ♀ and *Scomber scombrus* ♂ were made successfully during June and July, 1938 and 1939 at Woods Hole. The abnormality of the embryos, and the appearance of paternal pigmentation in the hybrid, as previously described by H. H. Newman, were confirmed.

A systematic re-investigation of the inheritance of pigmentation, followed by a cytological and morphological study of the hybrid seemed worthwhile.

Development of pigmentation in normal *Scomber scombrus* is described for the first time. Tables comparing the number of melanophores on embryos, and yolk, in parents and hybrids, seem to reveal a *Scomber*-effect in the hybrid.

As regards the chromatophores, the hybrid embryos appear to fall into three categories: those with *Scomber* type, those with *Fundulus* type, and those with both types of chromatophores. The melanophores and chromatophores in parents and hybrids are distinguishable: those in the hybrid being identical in color and structure with those of the parents. However, a close and systematic study of the hybrids reveals an enormous variability of combinations of the parental pigment cells. Actually, no two embryos are identical in their pigmentation pattern.

This enormous variation found in the F_1 generation is unusual, and at present inexplicable. The reciprocal cross was never successfully made.

The use of the swimbladder by fish in respiratory stress. Virginia Safford.

It is generally agreed that the chief function of the swimbladder is to maintain buoyancy in the fish. The use of oxygen in the swimbladder for respiration has been shown by Potter for physostomous fish (*J. Exp. Zool.*, **49**, 45, 1927). Physoclistous fish when confined in a limited volume also show gaseous exchange in the bladder without change in external pressure on the fish.

The scup, sea robin, cunner, tautog, fundulus and toadfish (all physoclisti) were bottled by the method of Fry and Black (*Am. J. Physiol.*, **126**, p. 497, 1939) in water with various pressures of CO_2. The water was analyzed for CO_2 and O_2 at the death of the fish and the results showed a characteristic curve for each species, *i.e.* the ability to use oxygen decreases with the increase in pressure of CO_2. At the same time gas samples were taken from the swimbladders and analyzed for CO_2 and O_2 by the use of Krogh's micro-gas-analyzer.

The analyses showed that for these species: (1) CO_2 passes freely into the swimbladder, the fish equilibrating with external CO_2 up to the point where his ability to use oxygen in the water decreases. (2) Oxygen in the bladder is used at low CO_2 pressures in the water but the ability to use oxygen in the bladder decreases with the rise in CO_2 in a manner parallel with the ability to use oxygen from the water.

Species differences occur in the curves showing the ability of the fish to use oxygen from water and swimbladder in the presence of CO_2. The ratio of CO_2 in the water and swimbladder is practically constant for each species, *i.e.* $\frac{\text{swimbladder } CO_2}{\text{water } CO_2} = k$, up to the point where utilization of oxygen in the water decreases. The values of k in these six species were not very different.

It seems, therefore, that the same sort of mechanism effects gaseous exchange between swimbladder and blood and between water and blood in the fish under the conditions of the experiments described.

Water permeability of Chaetopterus eggs. Herbert Shapiro.

The permeability of the egg of the annelid worm, *Chaetopterus pergamentaceus*, to water was determined by allowing the egg to swell in 60 per cent sea water. The equilibrium volumes of about thirty eggs were measured at various dilutions of sea water, and found to conform to Boyle's law. From the equation

$$P_0(V_0 - b) = P_{ex}(V_{eq} - b)$$

(where V_0 and P_0 represent respectively the egg volume, and osmotic pressure in normal sea water, and V_{eq} the cell volume at equilibrium in diluted sea water of osmotic pressure P_{ex}), the "osmotically inert volume," b, was found to be about 34 per cent of the cell volume. Individual plots were made of the kinetics of osmosis of 27 unfertilized eggs and of 21 fertilized eggs (at room temperatures, 22 to 25° C.). The permeability constant, K, was calculated for the first, third and fifth minute of swelling from the relationship proposed by Lucké, Hartline and McCutcheon (*Physiol. Rev.*, **12**, 68, 1932) viz.,

$$dV/dt = KS(P - P_{eq}),$$

where dV/dt represents the rate of change of egg volume at any instant, S the surface area of the cell, $P - P_{eq}$ the difference in osmotic pressure between the interior and exterior of the cell, and K the cubic micra of water passing across a square micron of cell surface per minute per atmosphere osmotic pressure difference. The "osmotically inert volume" of each egg was taken into consideration. These values for the unfertilized eggs obtained by direct microscopic measurement ($K = 0.5$) agree with those previously reported by Lucké, Ricca and Hartline (*Biol. Bull.*, **71**: 397, 1936) using a diffractometer method. It was also found that the permeability of the *Chaetopterus* egg to water shows little change on fertilization, the data indicating a slight increase ($K = 0.6$). Whitaker (*Jour. Gen. Physiol.*, **16**: 475, 1933) has demonstrated a decline in respiration of the *Chaetopterus* egg when fertilized. Thus it is evident that the parallelism between alterations in respiration and water permeability, which appeared on fertilization of the eggs of *Arbacia* and *Asterias*, is not a universal feature of the activity of marine eggs.

A mechanism of increased cell permeability resembling catalysis. M. H. Jacobs and A. K. Parpart.

It was shown by Ørskov that the rate of entrance of NH_4Cl into the mammalian erythrocyte is greatly accelerated by low concentrations of bicarbonates. This effect was attributed to an increased permeability of the cell to the NH_4 ion. The following is suggested as a more plausible explanation. NH_4Cl enters the erythrocyte by the penetration of NH_3 followed by the exchange of OH for Cl ions at a rate that depends on the value of $[OH]_i[Cl]_0 - [OH]_0[Cl]_i$. This rate is relatively slow because of the low value of $[OH]_i$. On the addition of bicarbonate a second penetrating molecule, CO_2, is formed, which for reasons discussed elsewhere (*J. Cell. Comp. Physiol.*, **7**, 351, 1936) leads to the accumulation within the cell of NH_4HCO_3 at a higher concentration than outside. An exchange of HCO_3 for Cl completes the entrance of NH_4Cl, the bicarbonate again being available to repeat the cycle. Because of the more favorable value of $[HCO_3]_i[Cl]_0 - [HCO_3]_0[Cl]_i$ the entrance of Cl under these conditions is far more rapid than before. In agreement with the theory, two parts of the swelling curve of the erythrocyte may be distinguished, the first, which involves only undissociated molecules, being little affected by butyl alcohol, the second, which depends on an ionic exchange, being strongly retarded. Even without alcohol two parts of the curve are apparent if cyanide or sulphide be substituted for bicarbonate. The ineffectiveness of acetates etc. seems to be due both to a slower entrance of free acid and to a less ready ionic exchange. The addition of bicarbonate to ammonium citrate gives only the first part of the swelling curve, since there is no penetrating anion externally for which HCO_3 can be exchanged. For reasons discussed previously (*loc. cit.*) even the initial swelling is absent when borates are used.

Oxygen consumption and cell division of fertilized Arbacia eggs in the presence of respiratory inhibitors. M. E. Krahl, A. K. Keltch, and G. H. A. Clowes.

For fertilized eggs of *Arbacia punctulata* initially exposed to the reagents at 30 minutes after fertilization at 20° C., the levels of oxygen consumption prevailing in the minimum concentrations of reagent producing complete cleavage block were: In 0.4 per cent oxygen—99.6 per cent nitrogen, 32 per cent of control oxygen consumption; in 0.7 per cent oxygen—99.3 per cent carbon monoxide, 32 per cent of control oxygen consumption; in 1.6×10^{-4} M KCN, 34 per cent of control oxygen consumption; in KCN at 24° C. the value was 16 per cent of the control oxygen consumption.

The carbon monoxide inhibition of oxygen consumption and cleavage was reversed by light from a powerful carbon arc lamp. The percentage of inhibition of oxygen consumption by CO in the dark is described by the equation $\frac{n}{1-n} \cdot \frac{pco}{po_2} = K$ where n is the fraction of oxygen consumption not inhibited, pco and po_2 the partial pressures of CO and O_2, respectively, and $K = 60$. A 20 per cent stimulation of oxygen consumption occurred in 10 per cent oxygen—90 per cent CO.

Spectroscopic examination of fertilized and unfertilized *Arbacia* eggs reduced by hydrosulfite revealed no cytochrome bands, although a band at 600–605 mμ corresponding to cytochrome *a* was found in *Arbacia* sperm. The thickness and density of the egg suspension used was such as to indicate that, if cytochrome is present at all, the amount in *Arbacia* eggs is extremely small as compared to that in other tissues having a comparable rate of oxygen consumption.

Three reagents poisoning copper catalyses, potassium dithiooxalate (10^{-2} M), diphenylthiocarbazone (10^{-4} M), and isonitrosoacetophenone (2×10^{-3} M) produced no inhibition of division of fertilized *Arbacia* eggs.

These results indicate that the respiratory processes required to support division in the *Arbacia* egg may be of a type not dependent on cytochrome for intermediate hydrogen transport, not dependent on a copper containing catalyst, and perhaps different in several essential steps from the principal respiratory processes of yeast or mammalian muscle.

Some factors affecting the rate of hemolysis of the mammalian erythrocyte by n-butyl alcohol. M. G. Netsky and M. H. Jacobs.

The hemolytic effect of solutions of *n*-butyl alcohol in isotonic NaCl varies greatly with small changes in concentration of alcohol and temperature. In certain parts of the range, concentration differences of 0.1 per cent or 0.0025 M butyl alcohol, and temperature differences of 0.25° C. are readily detectable. For the erythrocytes of eight species of mammals a maximum resistance to butyl alcohol hemolysis was found at pH 6.7–6.8, with times of hemolysis lower in the acid than in the alkaline range. The pH of the maximum lies near the isoelectric point of hemoglobin. If it be assumed that the alcohol causes a condition of cation-permeability of the erythrocyte, an effect for which there is already some independent evidence, the Gibbs-Donnan equilibrium demands a swelling of the cell on both sides of the isoelectric point; this might be expected to hasten the rate of hemolysis. Optical studies of volume changes made by the method of A. K. Parpart show that at a pH value of 8.60 hemolytic concentrations of butyl alcohol convert the usual shrinkage of the cells in alkaline solutions into a rapid swelling as demanded by the theory; swelling also occurs rapidly at pH 5.18, but very slowly at pH 6.70. Despite the similarity of the pH effect in different species, marked and characteristic specific differences were found in the absolute times of hemolysis. At the pH maximum, the order of resistance was:

monkey > man > dog > cat > rat > rabbit > beef > pig.

In accordance with the Gibbs-Donnan principle even low concentrations of non-penetrating non-electrolytes were found to cause a considerable retardation of alcohol hemolysis, but the effect proved to be more complex than a simple osmotic phenomenon, since with equimolecular concentrations of different non-electrolytes it was generally greater the greater the molecular weight of the added substance. This relation is seen in the following series for beef cells:

lactose > sucrose > mannitol > dextrose, levulose > xylose
> pentaerythritol, erythritol > malonamide.

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Studies on the permeability-decreasing effect of alcohols and pharmacologically related compounds on the human erythrocyte. J. B. S. Campbell and M. H. Jacobs.

In extending the work previously reported by Jacobs and Parpart (*Biol. Bull.*, **73**, 380, 1937), it was found that the hemolysis of human erythrocytes in 0.3 M solutions of glycerol at pH 7.4 is characteristically retarded by methyl, ethyl, *n*-propyl, *n*-butyl, *n*-amyl, *n*-hexyl and *n*-octyl alcohols, as well as by ethyl ether, chloroform, ethyl and phenyl urethanes and several other substances in a manner that in general runs parallel with their pharmacological activity. On reducing the pH below 6.0, substances of this type were found to give an acceleration rather than a retardation of hemolysis, as they do likewise with beef erythrocytes at all pH values studied. While the absolute time of hemolysis in 0.3 M glycerol solutions is more than doubled by lowering the temperature from 35° C. to 5° C., the minimal effective concentration of alcohol remains approximately constant over this temperature range. At body temperature, quantitatively measurable effects were obtained with ethyl ether, chloroform, and ethyl alcohol at concentrations considerably lower than those reported in the literature to exist in human blood in anesthesia and in alcoholic intoxication.

Quantitative studies of the rate of passage of protein and other nitrogenous substances through the walls of growing and of differentiated mammalian blood capillaries. Richard G. Abell.[1]

The rate of passage of nitrogenous substances through the walls of growing blood capillaries was observed with a transparent chamber, inserted in the rabbit's ear, called the "moat" chamber. In this chamber the growing capillaries can be seen with the high powers of the microscope, their condition recorded, and their area calculated. After passing through the walls of these capillaries, nitrogenous substances diffuse into a moat, or reservoir, of known volume, from which they can be removed and analyzed quantitatively.

(1) Analyses were made of the total nitrogen entering the moat during the first 24 hours following the introduction into the moat of a mammalian Ringer's solution. (2) The total surface of the capillaries involved was obtained from measurements of length and diameter. From these two sets of data the calculated amounts of total nitrogen passing through per sq. mm. of endothelial surface per 24 hours were, in 6 different chambers, as follows: (1) 0.091 mg.; (2) 0.113 mg.; (3) 0.102 mg.; (4) 0.097 mg.; (5) 0.046 mg.; (6) 0.081 mg.

The slower rate of passage of these substances (through the walls of the capillaries) in chambers 5 and 6 was associated with a slower rate of circulation in these two chambers, as observed with the microscope.

When the Ringer's solution was left in the moat for intervals of time longer than 24 hours, the total nitrogen content of the moat rose above the total non-protein nitrogen level of rabbit's blood within 5 days, which would seem to indicate that plasma protein passes through the walls of growing blood capillaries.

Analyses of the moat content for protein showed that this is the case. Using calculations similar to those described above in the one chamber containing blood capillaries in which such studies have so far been made (chamber 3), but basing the estimation on a collection period 48 hours in length, *protein* nitrogen came through the capillary wall at the rate of 0.039 mg. per sq. mm. of capillary surface per 24 hours. This compares with the figure for *total* nitrogen of 0.102 mg. Thus, of the *total* nitrogen that came through the walls of the growing capillaries in this chamber per 24 hours, approximately $\frac{1}{3}$ was *protein* nitrogen.

The results secured with a new type of chamber, the "filter disc" chamber, indicate that mature blood capillaries are permeable to protein, but less so than are growing blood capillaries.

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[1] Department of Anatomy, University of Pennsylvania School of Medicine.

August 30

The occurrence of cytochrome and other hemochromogens in certain marine forms. Eric G. Ball and Bettina Meyerhof.

The process of oxygen utilization in mammalian tissue appears to proceed through a chain of iron porphyrin compounds composed of hemoglobin, myoglobin, cytochrome oxidase and the three cytochromes *a*, *b*, and *c*. Certain marine animals, however, possess instead of hemoglobin a copper blood pigment, hemocyanin, which functions in a manner similar to hemoglobin. The question, therefore, arises as to whether these organisms are also deficient in those other iron porphyrin compounds that compose the respiratory chain in mammalian tissue. We have therefore examined the following hemocyanin-containing animals for these compounds: *Venus mercenaria*, *Busycon canaliculatum*, *Limulus polyphemus*, *Homarus americanus*, and *Loligo pealeii*. The heart and some body muscles of all these organisms were found to possess cytochrome oxidase and the three cytochrome components. In addition we have tested extracts of these same tissues for succinic dehydrogenase and have found that it is present in amounts which parallel roughly the concentration of cytochrome in these same tissues. Myoglobin has been found only in *Venus mercenaria* and *Busycon canaliculatum*. The radula muscles of the latter are extremely rich in this iron compound.

Two additional hemochromogens have been observed in *Limulus polyphemus*. One is present in the abundant clot obtained from the blood of the animal. Its reduced form possesses an absorption band centered at λ560 mμ. The oxidized form shows no characteristic band. The other is present in the eggs. Its reduced form shows an absorption band centered at λ625 mμ while the oxidized form has a band centered at λ570 mμ.

It would therefore appear that the process of oxygen utilization in these organisms whose blood pigment is a copper compound is similar to that in mammals except for the substitution of hemocyanin for hemoglobin. This substitution can therefore not be ascribed to the inability of these animals to utilize iron or to synthesize the porphyrin prosthetic group characteristic of the iron respiratory pigments.

The eggs and sperm of *Arbacia punctulata* were also examined. The sperm were found to contain abundant cytochrome oxidase, cytochromes *a*, *b*, and *c*, and succinic dehydrogenase. Tests for these same compounds in the eggs were negative. Upon addition of pyridine and sodium hydrosulfite to a ground egg suspension a strong hemochromogen band centered at λ560 mμ appeared indicating that a hemin is, however, also a constituent of the eggs.

Some observations on cholinesterase in invertebrates. Carl C. Smith and David Glick.

A study of the distribution of cholinesterase in some invertebrate hearts and in various tissues of *Limulus polyphemus* was made in an attempt to find some basis for explaining certain observations previously made concerning their reaction to cholinergic drugs.

The manometric method of Ammon utilizing the Warburg apparatus was used. In the following table the activities found are expressed in cubic millimeters of carbon dioxide produced per fifty milligrams of tissue per thirty minutes.

Invertebrate Hearts	
Modiolus demissus	304
Callinectes sapidus	51
Limulus polyphemus	50
Pagurus longicarpus	49
Libinia dubia	37
Venus mercenaria	5.5

Tissues of Limulus polyphemus	
Cardiac nerve	446
Ventral nerve	216
Heart (segments 5–6)	50
Heart (segments 1–2)	34
Blood serum	42
Blood cells (clot)	33
Intestine	18
Skeletal muscle	4.5

The concentration of cholinesterase found seems to roughly follow the amount of nervous tissue present. The enzyme was found in the blood and tissues of arthropods and crustacea in which it has previously been reported absent. The insensitivity of the clam heart to eserinization can be explained on the basis of the low esterase content found.

Crystalline myogen. Kenneth Bailey.

The albumin fraction of rabbit skeletal muscle is obtained in the form of long thin needles in the following way: the perfused minced muscle is treated with an equal volume of ice cold 1 per cent NaCl, and after standing for one hour is pressed dry. The globulin fractions in the press juice are separated by addition of solid ammonium sulphate until the specific gravity of the solution at 25° reaches 1.175, the pH being maintained at 6.3. After filtration the liquid is acidified with dilute H_2SO_4 to a pH of 5.8 and after standing overnight is again filtered. More ammonium sulphate is fed in with slow stirring through a collodion membrane, the crystals separating when the specific gravity reaches 1.18; at a specific gravity of 1.21 crystallisation is complete.

Effect of increased intracellular pH on the physiological action of substituted phenols. J. O. Hutchens and M. E. Krahl.

1. The effects of five substituted phenols (2,4-dinitrophenol, 4,6-dinitro-o-cresol, 2,4,5-trichlorophenol, 2,4-dichlorophenol, and m-nitrophenol) on the respiration and cell division of fertilized eggs of *Arbacia punctulata* have been determined at an extracellular pH of 8.0 in the presence and absence of a concentration of ammonia (0.004 M NH_4Cl) sufficient to increase the normal cytoplasmic pH from the normal value of 6.8 ± 0.2 to approximately 7.2 ± 0.2.

2. The following results were obtained:

a. The relative and absolute stimulation of oxygen consumption produced by suboptimum concentrations of each substituted phenol was greater in the presence than in the absence of ammonia.

b. The relative inhibition of cell division by each concentration of each substituted phenol was the same in the presence and absence of the ammonia.

c. The optimum respiratory stimulating concentration for each substituted phenol was the same in the presence and absence of the ammonia.

3. These results confirm and extend the experiments of Krahl and Clowes [*J. Cell. and Comp. Physiol.*, **11**, 1 (1938)] in which the cytoplasmic pH was decreased by means of carbon dioxide. Both the present and previous series of experiments, so far as the experimental and theoretical limitations of the method permit, indicate that the substituted phenol anion is the intracellular active form for respiratory stimulation and that the substituted phenol molecule is the intracellular active form for inhibition of cell division. The experimental data are completely inconsistent with the suggestion, advanced by Tyler and Horowitz [*Biol. Bull.*, **75**: 209 (1938)], that the substituted phenol anion is the intracellular active form for inhibition of cell division.

Fatty acid compounds in the unfertilized egg of Arbacia punctulata. Albert E. Navez.

In a note in this *Bulletin* (1938)[1] it was pointed out that some "oil" was reacting with the Nadi reagent (used in experiments on indophenoloxidase) concurrently with its reaction with the oxidase. Variable results in the saponification value and in the iodine number of this "crude oil" raised doubts on the adequacy of ether as an extraction fluid. Even carbon tetrachloride extractions showed differences unless the solvent was specially purified.

In the present experiments cyclohexane and CCl_4 were used as extraction solvents; in some cases after treatment of eggs (packed solidly by centrifuging) with alcohol, in others in an atmosphere of nitrogen.

In the "crude oil" sterols and phospholipids are present (cf. also Mathews, 1913;[2] Page, 1927[3]). This oil, dark red in color, heavy in consistency, with a strong fish oil odor and slowly semi-drying in thin films, was fractionated after saponification.

The fatty acids separated are: saturated fatty acids, unsaturated fatty acids and fatty oxyacids. Their relative quantities seem to be variable with successive batches of eggs and moment in the season. The largest portion of fatty acids are unsaturated; we have isolated by Br derivatives small quantities of the diethylenic, larger amounts of the triethylenic and definitely indications of tetraethylenic (clupanodonic?) in small quantities.

The red color of the oil is interesting as it can be removed by adsorption on norite but with concomittant removal of some fatty compound. By successive acid and alkaline treatments the red color can be eluted from the adsorbing agent; it gives the absorption spectrum of echinochrome in CCl_4. The possibility is seen of the presence of a fatty acid derivative of echinochrome, playing a possible rôle on oxidations in the egg, in view of the ease of oxidation of the unsaturated compounds.

The unsaponifiable as yielded—unfortunately in very small quantities—small crystals on treatment by HCl gas in anhydrous acetonic solution, which might point to the presence of some unsaturated hydrocarbon (squalene). No detailed work done due to small quantity available. From the present observations it appears that the composition of the "crude oil" is not constant but varies with time, state of animals, feeding, method and length of keeping, perhaps temperature of sea water. No correlation has been found. Work on a larger scale is planned for the future.

Color changes in luciferin solutions. Aurin M. Chase.

During the spontaneous non-luminescent oxidation of *Cypridina luciferin*, partially purified by Anderson's method, the visible absorption spectrum of the solution, which has initially a slightly increasing absorption toward the shorter wavelengths, rises, producing a maximum at about 470 mμ, and then subsequently falls. This change is much more rapid and its magnitude greater in aqueous solutions of luciferin than in butyl alcohol solutions and the loss of luciferin (as measured by light emission) in aqueous solutions and butyl alcohol parallels the color change.

Hydrogen peroxide causes very rapid and almost complete loss of color in luciferin solutions, together with very rapid oxidation of luciferin.

Measured at pH's from 5.1 to 10.2 the change in the absorption spectrum is much faster at alkaline than at acid pH's and so is the decrease in concentration of luciferin under these conditions, using the luminescent reaction as a measure of luciferin concentration.

These facts indicate that the color changes observed represent changes in luciferin itself.

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[1] Navez, A. E., 1938. *Biol. Bull.*, **75**: 357.
[2] Mathews, A. P., 1913. *Jour. Biol. Chem.*, **14**: 465.
[3] Page, I. H., 1927. *Biol. Bull.*, **52**: 164.

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Anderson has demonstrated that when luciferase is added to a partially oxidized luciferin solution approximately the same amount of light is emitted, but much more slowly, as is emitted when luciferase is added immediately upon dissolving the luciferin. To explain this he has postulated that the non-luminescent oxidation of luciferin is reversible and, in the presence of luciferase, the slowly emitted light in the former case is controlled by the reduction of this reversibly oxidized luciferin. This reversible oxidation is believed to be represented by the initial rise in the absorption spectrum of luciferin solutions during non-luminescent oxidation in air. The subsequent decrease in absorption must indicate another reaction, possibly also an oxidation; probably irreversible.

A change in the absorption spectrum of luciferin solutions also occurs as a result of the luminescent reaction itself, and this color change may perhaps be used in studying the luminescent reaction.

Photodynamic action in the eggs of Nereis limbata. Fred W. Alsup.

Photodynamic effects can be produced in the eggs of *Nereis* by exposing them in solutions of rose bengal or eosin of the proper concentrations to sunlight or to light from a 1000-watt electric bulb. The effects consist of: (1) elevation of wide membranes, (2) germinal vesicle breakdown and (3) cytolysis of the eggs. The percentage of nuclear breakdown varies with exposure time and the concentration of dye. Solutions of rose bengal stronger than 1 part to 20,000 parts of sea water produce effects on the eggs in the dark. These dark reactions involve nuclear breakdown, staining of the entire eggs and cytolysis. With eosin a much stronger concentration is required to produce the same degree of reaction. Previously irradiated solutions of rose bengal produce no observable effects on the eggs, but when eggs are put into previously exposed weak solutions of the dye and then removed to sea water and fertilized, many cleave irregularly or cytolyze, indicating that the previously exposed solutions did have some effect on the eggs. Most eggs develop normally in these weak solutions, if the solutions have not been exposed to light. Solutions of eosin previously exposed to light produce nuclear breakdown in the eggs. No photodynamic changes can be produced in the eggs in the absence of free oxygen. KCN increases photodynamic action on the eggs as shown by increased percentages of nuclear breakdown. Relatively concentrated solutions of rose bengal bleached by sunlight have little or no effect on the eggs, since eggs can be fertilized and will develop normally in such bleached solutions, whereas they can not develop in unbleached solutions of the same concentration.

The same general effects obtained with the eggs of *Nereis limbata* were obtained with the eggs of *Arbacia punctulata*.

Cleavage delay in Arbacia punctulata eggs irradiated while closely packed in capillary tubes. Irving Cohen.

From the work of Henshaw and others it is known that X-rays administered to *Arbacia punctulata* eggs prior to fertilization cause a delay in the occurrence of first cleavage.

Doctor Failla suggested to the writer the problem of comparing the radiosensitivity of these eggs when irradiated in the ordinary way and closely packed in capillary tubes.

The general technique worked out by Henshaw, (*Am. Jour. Roentgenol. and Rad. Ther.*, **27**, No. 6, June, 1932) has been followed in these experiments. Individual controls were set up for the two parallel series of experiments.

The results show that with equal dosages of radiation considerably less delay in cleavage is produced in the eggs irradiated in the capillary tubes. Doctor Failla predicted the result on the basis of his theory of the biological action of ionizing radiations (*Occ. Publ. Am. Ass. Adv. Sci.* No. 4, June, 1937). He has suggested that owing to the greater complexity of the molecules within the egg, the increase in ion concentration resulting from the X-rays would be relatively greater in the cytoplasm than in the sea water. Therefore the radiation would cause an initial ionic unbalance across the cell boundary and this is assumed to enhance the radiation effect. On the other hand, when the eggs are closely packed in capillary tubes there is practically no sea water around the cells and the ionic unbalance should be much less. Accordingly the radiation effect should be much less marked.

It should be noted that while the experimental results confirm Failla's prediction it does not follow that the suggested explanation is necessarily correct. (This point is fully appreciated by Failla who has followed the work with interest.) There are, of course, other possible explanations.

The X-ray effect on the cleavage time of Arbacia eggs in the absence of oxygen. Rubert S. Anderson.

From a chemical viewpoint it is possible to consider that one result of the ionization produced by the absorption of X-rays is the formation of products having oxidizing and reducing tendencies. Experimentally, a number of authors have found that oxidation or reduction is one of the types of reactions produced by X-rays in simple chemical systems. They are especially common in the dilute aqueous solutions studied by Fricke and Clarke where most of the reactions occur indirectly through the water molecules.

This same type of reaction almost certainly occurs within living cells during irradiation and it is important to know if it plays any significant part in biological effects. Skoog concluded that this was true for certain plants and Fricke has suggested similar possibilities for some genic effects in *Drosophila.*

In the present preliminary experiments the primary object has been to see if the X-ray effect could be modified experimentally, as a first step toward finding out what reactions are important. *Arbacia* eggs have been used. Henshaw showed that irradiation of the unfertilized egg increased the time from fertilization to the first cleavage. If oxidations or reductions produced by the X-rays are an important factor in this delay it seemed possible that irradiation in the absence of oxygen would modify the effect.

Oxygen was removed from suspensions of eggs in Thunberg tubes by washing out with purified hydrogen. These eggs and control eggs were irradiated with 15,000 r.

All of the eggs were fertilized in air within four minutes after the end of irradiation and the time until 50 per cent of the eggs had cleaved was determined.

In all of fifteen lots of eggs the delay in cleavage was greater for the eggs irradiated in air than for those irradiated in hydrogen and this increase in delay averaged about 50 per cent. However, the variability was very large and ranged from a low of 13 per cent, which is probably not outside the error, to over 100 per cent for eggs from different females.

The absence of oxygen (or conceivably the presence of hydrogen) therefore does modify this X-ray effect in most *Arbacia* eggs. This is not presented as an argument for the importance of oxidations or reductions but these results do encourage investigation in that direction.

Fixation of X-ray effect by fertilization in Arbacia eggs. P. S. Henshaw.

We have shown previously that exposure of *Arbacia punctulata* eggs to X-rays causes a delay in the occurrence of the first cleavage, and further, that if an interval of time is allowed between the end of treatment and the moment of insemination, the effect is reduced or lost as a function of time—the latter being a change referred to as *recovery.*

While these findings were satisfactory in demonstrating that recovery takes place so far as the first cleavage is concerned, they gave no information as to whether it is significant for subsequent cleavages and later development or whether it continues after fertilization. It is the purpose of this report to deal with these points.

The procedure followed was to treat a collection of eggs giving all the same exposure, fertilize samples of these at various times up to three hours after treatment, allow to develop for 48 hours, and then to classify the embryos as to degree of development. By the method used, it was possible to determine whether all samples had developed to the same extent and to compare the development in samples which had had different amounts of time for recovery to occur.

It was found first that development had proceeded farther in those samples which had been allowed the most time for recovery before fertilization; and secondly, that curves arranged to show the amount of development as a function of time, bore characteristic similarities to those based on delay in the occurrence of the first cleavage.

On the basis of these observations, therefore, it appears that the recovery from X-ray effect, which takes place before fertilization and is manifest by the time of the first cleavage, is significant in subsequent cleavage and later development; and consequently, that fertilization acts to fix whatever X-ray effect is present at the moment of fertilization.

Does the action of X-rays on the nucleus depend upon the cytoplasm?
William R. Duryee.

Germinal vesicles from small ovarian eggs of three local species of frogs were irradiated both in intact eggs and in the isolated condition in Ca-free Ringer. Dosages varied from 500 to 50,000 r.[1] Chromosomes in nuclei isolated after previous irradiation *in situ* showed progressive injuries starting from 1000 r, in contrast to those in isolated irradiated nuclei, which even after 50,000 r showed no marked differences from the controls. No appreciable latent period in any of the 28 experiments was observed. In support of the conclusion that radiation damage to the chromosomes results primarily from chemical products of the injured cytoplasm is the fact that nuclei, having first been isolated and then placed in a concentrated egg brei and exposed to 50,000 r, showed typical chromosome defects of nuclei irradiated *in situ*.

Chromosome injuries were of three types: progressive loss of side branches or chromomere loops (which I described here last summer), fragmentation of the longitudinal chromonemata, and frequent separation of the members of synaptic pairs. Contraction of the chromosomes occurred when they were exposed either to irradiated or to non-irradiated injured cytoplasm as previously described under the term *Autofixation*. These changes are distinct from simple displacements of the chromosome pairs from their normal central positions in the nuclear matrix which depend on other factors.

PAPERS READ BY TITLE

Moulting and viability after removal of the eyestalks in Uca pugilator.
R. K. Abramowitz and A. A. Abramowitz.

Operative mortality following eyestalk removal in *Uca* is about 8 per cent for the first 24 hours. Eighty animals were isolated in paper cups (to abolish cannabalism) on the second day following eyestalk extirpation, and 25 normal animals were kept as controls under identical conditions. After one month, 12 per cent of the operated animals died directly without moulting, whereas none of the normal animals died without moulting. Moulting in the operated animals began on the tenth day, the percentage increasing rapidly as a hyperbolic function of time. Fifty per cent of the animals had moulted by the sixteenth day, and 96 per cent by the twenty-eighth day. Only 5 of the normal animals had moulted by the end of a month beginning the twenty-fifth day. The operated animals experienced considerable difficulty in moulting, many of them dying during or shortly after moulting. The total mortality was 74 per cent at the end of the month, 62 per cent being due to death in moulting. Forty per cent of the normal animals that moulted died during moulting. Blinded animals appear to lose pigment, an effect which becomes especially evident after moulting.

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[1] Irradiation experiments carried out by E. LP. ittle.

A new method for the assay of intermedin. A. A. Abramowitz.

The proposed method is based on previous observations that a pale denervated caudal band cannot darken during black-adaptation in hypophysectomized *Fundulus*, and that maximal sub-lethal doses of purified intermedin evoke a darkening in the denervated band but nowhere else in the integument of white-adapted fishes. In the caudal fins of 400 *Fundulus*, a 2 mm. band was made and the fishes white-adapted for 5 days, at which time both innervated and denervated regions of the tail were uniformly pale. In one series, graded doses of purified intermedin and in another, weighed samples of commercial pituitary powder (sheep whole gland) emulsified in distilled water were injected intraperitoneally into 20 fishes for each dose. The percentage of animals which responded was determined after a half-hour. The points fall on a smooth hyperbolic curve whose steepest part lies between 0 and 40 per cent. A unit of activity is defined as that amount of intermedin which darkens the denervated band in 25 per cent of the animals, at least 20 animals being injected. This test, in addition to being quantitative, seems to be quite specific. Twenty drugs, mainly alkaloids, were tested in various dosages, and all were ineffective in producing this reaction. In fact, the drugs usually darken the entire integument, leaving the pale band unaffected—an effect diammetrically opposite to that of pituitary intermedin.

Analysis of the electrical discharge from the cardiac ganglion of Limulus. Florence Armstrong, Mary Maxfield, C. Ladd Prosser, and Gordon Schoepfle.

The median ganglion of the *Limulus* heart contains two types of nerve cell, large unipolar ganglion cells found in segments 4 to 7 and small multipolar cells found in the outer portion of the whole nerve cord (Heinbecker, A. J. P., 1933, 1936). In action potential records of the activity of isolated portions of this ganglion we find no spontaneous discharge from segments 1, 2, 3, and 8. Rhythmic cardiac discharges occur in the intermediate segments. Occasionally, particularly in segments 5 to 7, there is a continuous spontaneous background upon which the rhythmic cardiac bursts are superimposed. Low potassium tends to favor the asynchronous background activity.

It has been postulated (Heinbecker et al.) that the small neurones are activated by the large pacemaker cells. A 0.1 per cent solution of nicotine, which abolishes all reflex activity in the central nervous system of the animal, was applied to the cardiac ganglion. The duration of the bursts remained constant, the interval between bursts diminished, thus increasing the cardiac frequency. This result indicates that activation of small neurones by the large ones is not by way of synapses.

Analysis of the activity in ganglia dissected down to a very few fibers showed that some neurones discharge only once per heart beat. Others discharge many times at a declining frequency during the burst. The cells providing the spontaneous background fire at a relatively constant frequency.

The intra-cellular distribution of reducing systems in the Arbacia egg. Robert Ballentine.

A comparison of the distribution of reducing activity (the dehydrogenase systems) with the cellular constituents has been studied by the manometric method of Quastel and Wheatley (1938) in centrifugally fragmented and whole *Arbacia* eggs. Allowing for considerable individual variation from urchin to urchin, it may be stated that the sum of the activities of the two halves is greater than that of the unfertilized egg, and approximately equivalent to that of the intact fertilized egg. Centrifugal and osmotic stretching of the egg, provided it is sub-threshold for activation, has little or no effect on the dehydrogenase systems. Since sedimentation of granules similar to that obtained in the half eggs is present in the centrifugally stretched cells, one is led to the conclusion that as far as the dehydrogenases are concerned, the process of fragmentation is equivalent to activation, although the halves are not induced to parthenogenetic development nor do they develop membranes. The exposure to 0.95 M sucrose, as employed in fragmentation, is without effect. On the basis of equivalent volumes, the red half has a greater activity than the white half, thus definitely indicating that the dehydrogenating systems are not exclusively limited to the clear cytoplasm, as is the peptidase activity (Holter, 1936). Rather it leads to the conclusion that a considerable portion of the substrate activation occurs at heterogeneous phase boundaries between the granules and the clear cytoplasm, or perhaps within the granular material itself.

Some effects of colchicine upon the first division of the eggs of Arbacia punctulata. H. W. Beams and T. C. Evans.

At room temperature 0.0002 molar concentration of colchicine in sea water inhibits cleavage of *Arbacia* eggs when applied at any time before (approximately) ten minutes of the appearance of the first cleavage furrows. Eggs left in this solution for one hour, washed and let stand in sea water show recovery as indicated by the appearance of cleavage furrows, many of which are abnormal. Eggs which have started to cleave when put into the colchicine solution continue the process until the two blastomeres are formed. However, all further cleavages are suppressed unless the colchicine is removed.

Eggs placed in the colchicine solution ten minutes after fertilization and centrifuged ten minutes later show a more marked stratification than do controls centrifuged for the same time and speed. Likewise, they show more stratification than do controls of the same lot fertilized ten minutes later and which are in approximately the same stages of division as were the experimental eggs when placed in the colchicine solution.

These results indicate that colchicine in concentrations sufficient to block cleavage acts by lowering the viscosity; or by inhibiting the normal rise in viscosity (gelation) that is associated with the appearance of the mitotic apparatus of the cleavage process. This is further substantiated by the fact that a disintegration of the asters may be observed when the eggs are placed in the colchicine approximately ten minutes before the appearance of the cleavage furrows. The rays seem to fade out, leaving only clear and often irregular areas in the position formerly occupied by the asters.

Temporal summation in neuromuscular responses of the earthworm, Lumbricus terrestris. E. Frances Botsford.

Temporal summation has been demonstrated in vertebrate smooth muscle and in the muscle of crustaceans and coelenterates. The phenomenon in smooth muscle of vertebrates has been attributed to the spreading of a chemical mediator through the tissue so that with each additional stimulation more muscle fibers contract. This study is to demonstrate temporal summation in the muscles of the body wall of the earthworm and the dependence of this summation upon a chemical mediator.

The earthworm was arranged for recording the contractions of the longitudinal muscles by a weak isometric lever. Stimulating the nerve cord at 8-second intervals with a tetanizing current of constant intensity and brief duration produced a facilitation of the successive responses, in some cases for as many as sixteen times. The dependence of the magnitude of the response upon the frequency of stimuli was demonstrated conclusively by varying the frequency by means of a vacuum tube stimulator. Furthermore, at constant frequency there was an increase in response with increase in duration of the stimulation. Thus it is evident that the strength of the response is dependent upon the number of stimuli and temporal summation is shown to be characteristic of the muscle responses of the earthworm.

Since this summation was also true of a dorsal muscle strip, the phenomenon is not dependent upon the nerve cord, but is produced either in the peripheral plexus or at the neuromotor junction.

The dependence of temporal summation upon the chemical mediator acetylcholine was demonstrated as follows:

(1) Eserine caused no response in an unstimulated dorsal muscle strip, but electrical stimulation of an eserinized muscle strip caused an increase in tension similar to that produced by acetylcholine.

(2) When the interval between stimulations was increased to 5 minutes there was no summation of the successive responses. But when eserine was applied to the muscle summation occurred in spite of the long interval between stimulations.

Since the muscle of the earthworm is very sensitive to acetylcholine, these experiments indicate that temporal summation is brought about by the spread of acetylcholine from the neuromotor junctions.

The source of chromatophorotropic hormones in crustacean eyestalks. F. A. Brown, Jr.

Through a series of injection experiments in which extracts of whole eyestalks and certain portions of eyestalk tissue were injected into *Palaemonetes* and *Uca* as test animals it has been demonstrated that the active source of chromatophorotropic hormones is a small, translucent, or bluish white mass located in the dorsal or dorsolateral region of the eyestalk. This tissue appears to be the sinusgland of Hanström and constitutes a definite gland which can dissected out easily in the forms investigated: *Cambarus*, *Carcinus*, *Callinectes*, *Libinia*, *Uca*, *Pagurus*, *Crago*, and *Palaemonetes.* In the last two named forms the gland is readily visible in the normal living animal. Quantitative studies of the effects of extract of the gland show that better than 80 per cent of the activity of the whole eyestalk extract is to be found in extracts of the minute gland. The difference of about 20 per cent can be accounted for by diffusion of substance from the gland into other eyestalk regions. Furthermore, the effect of the sinusgland by itself is qualitatively indistinguishable from that of the whole eyestalk of the same species judging by the relative effects of the sinusgland and whole eyestalk extracts upon the red chromatophores of *Palaemonetes* and the black ones of *Uca*. Implantation of the sinusgland of *Carcinus* into the abdomen of eyestalkless *Palaemonetes* has given confirmation of its chromatophorotropic activity as a single implant maintained the red pigment of *Palaemonetes* more or less concentrated over a five-day period at the end of which time the gland apparently became functionless.

Comparative effects of sinusgland extracts of different crustaceans on two chromatophore types. F. A. Brown, Jr. and H. H. Scudamore.

Extracts of the sinusglands of seven crustaceans (*Callinectes*, *Carcinus*, *Libinia*, *Pagurus*, *Uca*, *Crago*, and *Palaemonetes*) were each tested simultaneously on the red chromatophores of eyestalkless *Palaemonetes* and the black chromatophores of eyestalkless *Uca*. The order of effectiveness of the different sinusgland extracts upon the two chromatophore types were not the same, some extracts having relatively more effect upon the *Uca* black and others upon the *Palaemonetes* red chromatophores. This was expressed in the form of a ratio:

$$\frac{\text{Effect upon } \textit{Uca} \text{ black chromatophores}}{\text{Effect upon } \textit{Palaemonetes} \text{ red chromatophores}} .$$

The values of this ratio when sinusglands alone were used showed a definite sequence which was substantially repeated when whole eyestalks were used. Of the crustaceans examined *Crago* showed the highest value for the ratio while *Palaemonetes* and *Callinectes* showed the lowest. To support an hypothesis that two hormonal substances are to be found in different proportions in the various sinusglands, the discovery was made that dried sinusglands, or eyestalks, extracted with absolute ethyl alcohol yielded a fraction with a very low value for the above-mentioned ratio while a sea-water extract of the alcohol-insoluble residue yielded a fraction with a very high value. Thus, there seems to have been effected a partial separation of two chromatophorotropic principles from the crustacean sinusgland.

On the control of the dark chromatophores of Crago telson and uropods. F. A. Brown, Jr. and H. E. Ederstrom.

Since Koller's work indicating the presence of a rostral organ secreting a pigment-dispersing hormone there has been general lack of confirmation and even denial of its actuality. An exhaustive series of injection experiments in which extracts of various regions and tissues of the body of *Crago* were injected into eyestalkless *Crago* has disclosed what appears to be the normal source of a dispersing humoral substance for the dark pigment of the telson and uropods. The only tissue of the many tried which yielded darkening of the tail in practically 100 per cent of the trials was the central third of the circum-oesophageal connectives including the connective ganglion and a short portion of the connectives immediately posterior to the ganglion. From eyestalkless and black-adapted animals this middle third was usually the only effective region but in the case of white-adapted animals frequently all three portions of the connectives (anterior, middle, and posterior), and often even the posterior portion of the brain were somewhat active. This last was probably due to the diffusion of substance through the connectives. An extract of *Palaemonetes* connectives acts similarly upon *Crago*, though extracts of connectives of *Carcinus*, *Uca*, *Libinia*, *Pagurus*, and *Callinectes* fail to produce the response. The dispersing action of the connective hormone is annulled by extracts of eyestalks of *Crago* or *Palaemonetes* but is apparently uninfluenced by eyestalk extracts of *Carcinus*, *Libinia*, or *Uca*. The latter extracts exert the interesting effect of blanching strongly the trunk and leaving the black "tail" more conspicuous than ever, by contrast. The observations thus indicate definitely that there is an extra-eyestalk origin of a chromatophorotropic hormone and that *Palaemonetes* and *Crago* have a principle in their eyestalks not to be found in the eyestalks of *Carcinus*, *Libinia*, and *Uca*.

Micromanipulation of salivary gland chromosomes. John B. Buck.

Micromanipulation of normal salivary gland chromosomes of *Chironomus plumosus*, *in vitro*, proved infeasible because dissection of the cells causes immediate and marked abnormalities in the chromosomes. However, immersion of the gland in a hanging drop of isotonic Ringer's over the vapor of osmium tetroxide for 15 to 18 hours at 5° C. renders the cytoplasm and nuclear membrane sufficiently brittle so that individual chromosomes can be isolated. These chromosomes resemble those *in vivo* very closely in regard to minute visible structure and dimensions, and in addition partly retain the power of living chromosomes of responding reversibly to osmotic changes in their environment. The mechanism of action of the osmic vapor is obscure, but apparently involves a surface reaction, rather than impregnation, since the principal effect is loss of stickiness, and no osmium could be found inside the chromosomes following reduction.

The following results were obtained from manipulation of these chromosomes:

(1) A photographic record was obtained of the stage-by-stage reversible transformation of staggered transverse rows of achromatic droplets (honeycomb) into longitudinal parallel thread-like striations, supporting Metz's view that the latter are artifacts.

(2) Most of the longitudinal stretching occurs in the interband regions. Return to original length in relaxation may occur after up to 300 per cent stretch, and at least 500 per cent stretch may be sustained before breakage occurs. Breakage always occurs in the interband regions and in a straight line at right angles to the long axis of the chromosome.

(3) The somatically synapsed homologs are so intimately fused that forces sufficient to break the chromosome cannot separate them.

Effects of Roentgen radiation on certain phenomena related to cleavage in Arbacia eggs (Arbacia punctulata). T. C. Evans and H. W. Beams.

Fertilized eggs irradiated at 7,400 r/m. (minute exposures with a maximum dosage of six) showed subsequent delay in first cleavage which increased exponentially with the dosage.

Clumping of the eggs was noticed in the irradiated lots and it was found (by staining with Janus green) that the jelly, which surrounded the control eggs, was missing in the irradiated lots. Absence of the jelly was also noted in irradiated lots of unfertilized eggs. The effect was noticed immediately after irradiation and appears to be a direct radiation action as eggs placed in irradiated sea water were found to retain their enveloping jelly as long as did eggs in untreated water. Dosages below 10,000 r were not completely effective. Some fertilized eggs were supported in a hanging drop over polonium and were examined at the time when the controls were in the eight-cell stage. Some of the eggs in the drop were apparently not affected but some of them showed unilateral delay in cleavage. Such eggs stained with Janus green possessed the jelly only on the side of the unaffected blastomeres.

Irradiated sperm were found to lose their motility and fertility more rapidly than did the controls. Irradiated sperm (radiation sufficient to delay subsequent cleavage of eggs fertilized with treated sperm) were found to produce the initial rapid increase in oxygen consumption, as noted for controls, upon fertilization. Dead sperm, when added to the egg suspension in the respirometer, failed to produce the increased oxygen uptake. It appears that the rapid increase in oxygen consumption noted in normal fertilization may be related to the entrance of the sperm (or other related surface actions) regardless of the subsequent fate of the sperm nucleus.

Fertilized eggs irradiated as high as 37,000 r showed less stratification than controls when centrifuged at the same time.

The above findings are apparently unrelated in the present preliminary state of the investigation. It is evident, however, that radiation may produce several quite different immediate biological effects in the same cell and that such effects must be considered in attempting to formulate any possible fundamental biologic action of radiation.

Chemical and mechanical properties of two animal jellies. John D. Ferry.[1]

The jelly surrounding the eggs of *Arenicola cristata*, when dialyzed free of salts, contained only 0.2 per cent solid matter. It could be reversibly shrunk by concentrated salt solutions or by alcohol, acetone, or dioxane; it was not dissolved by zinc chloride, urea, or dilute acid. Qualitative tests showed that the material was a polysaccharide containing uronic acid. The jelly was precipitated by barium chloride (thereby releasing the eggs) in the form of long fibers, which swelled only slightly in cold dilute acid or salt solutions.

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[1] Society of Fellows, Harvard University.

The relaxation of shearing stress in the dialyzed jelly, shrunk to different extents, was examined in a concentric cylinder apparatus. Analysis of the decay curve indicated two relaxation times of the order of 2 and 100 minutes respectively, which decreased somewhat with increase of temperature, but showed little change in a concentration range where the rigidity varied tenfold.

Fresh specimens of *Mnemiopsis leidyi*, drained of excess sea water, were treated with borate buffer at pH 9.5 to dissolve the softer parts, and the residue discarded. When the solution was brought to pH 3, a viscous gel formed, and separated as a gelatinous, stringy clot. This was compressed by centrifuging and extracted with alcohol, thereby removing a small quantity of yellow lipoid material. The clot was resuspended in water and washed free of alcohol.

Qualitative tests on the material thus purified showed it to be a mucoprotein, containing 12 per cent nitrogen, and small amounts of sulfate sulfur and cystine. It swelled increasingly with increasing pH, dissolving alkaline to pH 8. In the neighborhood of pH 8 it formed highly viscous gels; this property was, however, destroyed by concentrated urea or by boiling.

The relaxation of shearing stress in a 0.1 per cent solution in M/6 borate buffer at pH 8.8 showed a relaxation time of the order of 2 minutes, which decreased with increasing temperature.

Response of frog striated muscle to $CaCl_2$. Judith E. Graham and F. J. M. Sichel.

The local application of $CaCl_2$ to the surface of a length of isolated skeletal muscle fibre causes a marked reversible shortening of the muscle substance, as pointed out by Chambers and Hale and by Keil and Sichel. This behavior of the isolated length of fibre is in marked contrast to that of the intact muscle, where no such shortening occurs even with concentrations of $CaCl_2$ as high as 400 mg. per cent. Since KCl-treated muscles have been shown by one of us to resemble in some respects the isolated fibre preparation, it was thought advisable to investigate the possibility that such muscles might behave like the isolated fibre also with respect to $CaCl_2$.

The isolated fibre shows no marked response to the injection or local application of KCl. The intact muscle (frog) when placed in a modified Ringer's solution containing 400 mg. per cent KCl undergoes a rapid transient contracture which disappears in 3 to 5 minutes. Subsequent to this treatment of the muscle with the KCl, and its response, the muscle will shorten markedly if placed in a similar solution containing 400 mg. per cent $CaCl_2$. This shortening is reversibly maintained in the presence of the excess $CaCl_2$ if the excess KCl is still present in the solution, but subsides slowly if only the normal concentration of KCl is present. This sensitization of the muscle to $CaCl_2$ by excess KCl is reversible; that is, washing the muscle in Ringer's solution after the transient KCl contracture renders it insensitive again to the action of $CaCl_2$. No antagonism of the $CaCl_2$ contracture by an equal KCl concentration could be detected.

Curarized muscle in Ringer's solution, and muscle in isotonic sucrose behaves like the KCl-treated muscle.

The permeability of the toadfish liver to inulin. Charlotte Haywood.

An earlier investigation (Haywood and Höber, *Jour. Cell. Comp. Physiol.*, **10**, 305, 1937) has indicated that the relatively large, lipoid-insoluble inulin molecule penetrates the isolated bullfrog liver, from perfusion fluid to bile, as through a passive filter. The present study demonstrates that in a living, unanaesthetized animal, retaining its normal blood supply to the liver, administered inulin can also enter the bile.

The toadfish was used because its aglomerular kidney fails to eliminate inulin. (Shannon, *Jour. Cell. Comp. Physiol.*, **5**, 301, 1934); 1.5 to 2. grams inulin per kilogram of body weight were injected intramuscularly a day or more before collection of bile samples. During collection, fish were strapped down and kept alive by a stream of water entering the mouth and directed over the gills. A ventral incision was made, the bile duct ligated, the gall bladder drained and cleaned, and a cannula inserted, after which the incision was closed, leaving the cannula protruding. Such preparations secreted up to 3 or 4 mg. bile per hour per gram of liver over a collecting period of 11 to 21 hours, after which a blood sample was drawn, usually from a caudal vessel, occasionally from the heart. Survival of fish following the operation was obtainable, often for several days.

Twenty-fold dilutions of bile and of protein-free blood plasma were analyzed by the Shaffer-Somogyi method for reducing substance. The difference in reducing substance before and after hydrolysis with H_2SO_4 represents the amount of inulin hydrolyzed to levulose.

A series of nine experiments showed the concentration of inulin in the bile to range between 55 and 73 per cent of that in the blood plasma. These figures are corrected for a small amount of reducing substance found in the bile in control experiments, without inulin.

A further series to determine the effect of a choleretic is under investigation.

Activation of Cumingia and Arbacia eggs by bivalent cations. Josephine Hollingsworth.

Eggs of *Cumingia tellinoides* are activated by isotonic solutions of $SrCl_2$, $CaCl_2$ and $BaCl_2$. The degree of effectiveness of the various salts follows the order named. The various pHs from 6.1 to 8.6 are equally favorable for activation. The time of polar body formation in eggs activated by bivalent cations is approximately the same as the time of polar body formation in eggs activated by sperm. The addition of sea water or the addition of isotonic solutions of NaCl or KCl tends to inhibit activation by bivalent cations. This inhibiting effect increases as the proportion of sea water or the concentration of the monovalent cations increases.

Eggs of *Arbacia* are activated by isotonic solutions of $CaCl_2$, $SrCl_2$, $MgCl_2$ and $BaCl_2$. Calcium ions are more effective than strontium, magnesium and barium ions. The action of the last three cations is somewhat variable. Whereas the bivalent cations act rapidly on *Cumingia* eggs, *Arbacia* eggs must be exposed to them for hours before any effect is observed. In eggs exposed to calcium ions, there is a much higher percentage of cleavage in ovary eggs which have been washed than in ovary eggs which have not been washed; in shed eggs than in ovary eggs either washed or unwashed; and there is a slightly higher percentage of cleavage in shed eggs that have been washed than in shed eggs that have not been washed. The highest percentage of cleavage takes place at pH 9.0. Below pH 8.8 the percentage of cleavage is usually small and above pH 9.2 the percentage of cytolysis is large. Whereas the addition to the calcium solution of isotonic solutions of NaCl or KCl tends to inhibit the activation of *Cumingia* eggs, in certain proportions the addition of these solutions to the calcium solution may increase the percentage of activation of *Arbacia* eggs while in other proportions the addition of these solutions has the opposite effect.

The vacuole systems of a fresh water limacine Amoeba. Dwight L. Hopkins.

By means of high power apochromatic objectives and staining with Nile blue sulfate, neutral red and Janus green, each type of vacuole and granule found in this amoeba has been followed from its origin to its disappearance. In general there are two systems of vacuoles. In active feeding amoebae the vacuoles arise from the protoplasm. One set of vacuoles, by swelling and coalescence, form the "contractile vacuoles" which are periodically evacuated to the outside. A second set of vacuoles coalesce with engulfed food and form the food vacuoles which after digestion has taken place are evacuated to the outside. Evacuation of the food vacuoles generally is independent of the contractile vacuoles. Occasionally, however, a food vacuole may coalesce entirely with a contractile vacuole and then this resulting food-contractile vacuole is evacuated. Again, the fluid portion of the food may run into the contractile vacuole leaving the food residue practically in contact with the protoplasm. Following this the food residue is soon evacuated but independently of the contractile vacuole.

In feeding or slowly feeding amoebae granules stainable with neutral red, Janus green B, and Nile blue sulfate arise in the protoplasm instead of food vacuoles. Under certain conditions more abnormal than those which cause cessation of feeding the contractile vacuole system is retarded and granular structures stainable with Janus green B, but not with Nile blue or neutral red, replace the fluid vacuoles which form the contractile vacuoles. Under favorable conditions the contractile vacuole system is not conspicuously stained with Janus green B. At a certain intermediate stage granulation of the contractile vacuole system becomes stainable with Janus green, but still these Janus green stained vacuoles can be observed to coalesce, swell and form the contractile vacuole in which a greenish-blue tinge definitely can be detected.

Cytological studies on androgenetic embryos of Triturus viridescens which have ceased development. Cornelius T. Kaylor.

Fankhauser (*J. E. Z.*, **68**, 1934) has shown that there is a high death rate in developing egg fragments of the European newt, *Triton palmatus*, during blastula and gastrula stages and that this is caused by the presence of irregular numbers of chromosomes in the cells of the embryos. A correspondingly high mortality during these same stages of development was observed in my experiments on androgenesis in eggs of *Triturus viridescens* (*J. E. Z.*, **76**, 1937). It was, therefore, reasonable to expect that the same abnormal chromosomal conditions as were found in *Triton palmatus* egg fragments would be responsible for the death of these androgenetic embryos of *viridescens* during the blastula and gastrula stages.

In a study of about 65 blastulae and 8 irregular gastrulae fixed at cessation of development, it was found that the cells of all these embryos were equipped with subhaploid to superhaploid numbers of chromosomes. The cessation of development was substantiated by the onset of cytolysis in all these cases. In a preliminary study of chromosome numbers in more advanced embryos which have ceased development, that is, neurulae and tail bud stages, it was found that there were only four questionable counts in over 100 which showed other than the haploid number of chromosomes.

It appears, then, that at least the complete haploid set of chromosomes is necessary in androgenetic embryos of *Triturus viridescens*, the same as in merogonic *Triton palmatus* embryos, if they are to develop beyond gastrulation.

Also a large number of mitoses were found in androgenetic embryos of *T. viridescens* fixed while still developing, which had no chromosomes at all on the spindle. Apparently cell division can proceed in the *T. viridescens* egg as well as in *Triton palmatus*, in the absence of chromosomes.

Effect of leukotaxine on cellular permeability to water. Valy Menkin.

The writer has recently succeeded in demonstrating the presence of a crystalline nitrogenous substance from inflammatory exudates capable *per se* of increasing capillary permeability and of inducing the migration of polymorphonuclear leukocytes. The liberation of this substance offers a reasonable explanation for two of the basic sequences in the development of the inflammatory reaction (*J. Exper. Med.*, 1936, **64**, 485 and 1938, **67**, 129, 145). This substance has been named *leukotaxine*.

In an endeavor to determine the mechanism of action on individual cells, the effect of leukotaxine on the permeability of ova of *Arbacia punctulata* was studied. Ova were exposed for intervals varying from 20 minutes to about an hour and a half to concentrations of leukotaxine ranging from about 3 to 8 mg. per cc. The eggs were subsequently immersed in hypotonic sea water (50 per cent concentration) and their degree of swelling measured from minute to minute with an eyepiece micrometer. A large number of ova were thus studied for a total period of 6 to 8 minutes. Their mean diameter served as a basis for the calculation of cell volume as previously described in the various contributions of Lucké and McCutcheon (cf. *Physiol. Rev.*, 1932, **12**, 68). The permeability was also obtained from the equation utilized by these workers: Permeability $= dV/dt/S(P - Pex)$.

The duration of exposure to leukotaxine prior to immersion in the hypotonic medium was found to be relatively inconsequential provided the pH had previously been adjusted to approximate that of sea water. A failure to follow this precaution might ultimately induce a change in ova exposed for long intervals to an acid pH that tends to reduce their swelling capacity when placed in a hypotonic medium.

The results of several experiments on a considerable number of ova indicate that leukotaxine appreciably increases the permeability of *Arbacia* ova to water. The extent of augmented permeability over that found in the case of normal ova in a similarly hypotonic medium is about twofold.

Effect of leukotaxine on cell cleavage. Valy Menkin.

Leukotaxine induces increased capillary permeability and migration of polymorphonuclear leukocytes in mammalian tissue (Menkin, *Physiol. Rev.*, 1938, **18**, 366). Its effect on ova of *Arbacia punctulata* is to enhance further their permeability to water when immersed in a hypotonic medium.

Does leukotaxine induce sufficient injury to ova to influence cleavage development? A series of observations have yielded the following results, summarized in brief:

1. Leukotaxine-treated ova manifest the usual fertilization reaction when exposed to sperms. The fertilization membrane, however, appears as a distinctly narrower zone than is seen under normal circumstances.
2. An appreciable number of fertilized ova fail to segment when exposed, for even a few minutes prior to fertilization, to a solution of leukotaxine.
3. The rate of cleavage of leukotaxine-treated ova tends to be retarded.
4. In the leukotaxine-treated group a considerable number of dividing eggs reveal atypical forms exemplified by unequal cleavage.
5. Sperms immersed for about an hour in sea water containing leukotaxine fail to fertilize normal ova.

These results indicate that leukotaxine seems to be definitely injurious to isolated cells as exemplified in the ova and sperms of *Arbacia punctulata*. In the case of ova this is manifested by increased permeability to water and by an appreciable inhibition to normal cleavage.

Response of the Arbacia egg cortex to chemical and physical agents in the absence of oxygen. Floyd Moser and J. A. Kitching.

Previous attempts to determine whether membrane elevation can be initiated in the *Arbacia* egg in the absence of oxygen have failed because of the fact that the sperm is immobilized under these conditions. Thus there is no proof that the *Arbacia* egg cortex could not respond in the complete absence of oxygen if it were given an adequate stimulus. In the present experiments mechanical and chemical agents have been used, to avoid the effect of oxygen lack upon the sperm.

Drops of *Arbacia* eggs and of the various agents required were suspended from a cover slip sealed with vaseline over a modified Englemann gas chamber. A monolayer of ferric stearate (see Ballentine, *Science*, **89**, 1939) was laid down on the cover slip to prevent coalescence when several drops were employed. A short, wide, bent, mercury-filled, glass side-arm allowed gas-tight manipulation of a microneedle or fine glass loop within the chamber. Oxygen-free hydrogen was passed through the chamber at 50–75 cc. per minute, and adequate time was allowed for equilibration. Mixing of the drop was achieved by slight shaking of the chamber, and transfer of the eggs with little fluid from one reagent to another was accomplished by means of the loop.

Eggs treated with saponin (1/4 of 1 per cent, in sea water), molar urea, and molar sucrose solutions in the absence of oxygen exhibited the same characteristic cortical response and membrane elevation as that obtained in air (see Moser, *J. E. Z.*, **80**, 1939). Moreover, the time relationships were much the same, regardless of whether the experiments were made in an atmosphere of hydrogen, carbon-dioxide-free air, or air. Shortly after the response to the non-electrolyte solutions in the absence of oxygen, the elevated membranes disappeared (see Moser, *Biol. Bull.*, **73**, 1937). Typically the response to saponin was followed some minutes later by cytolysis. The response to pricking with a microneedle under anoxic conditions was not unlike that obtained in air (see Moser, *J. E. Z.*, **80**, 1939).

Eggs transferred under anoxic conditions to hypertonic sea water exhibited cortical alveolar swelling (see Hunter, *J. C. C. P.*, **9**, 1936), but no membrane elevation. When, however, the eggs had previously been treated with urea, which itself initiates the cortical response, no such cortical alveolar swelling took place.

When, by means of the loop, eggs were transferred in the absence of oxygen first to urea, and then through several changes of sea water, they subsequently cleaved when exposed to air, but did not cleave when kept under anoxic conditions. Eggs left in urea undergo amoeboid movements in air (Moser, unpublished observations), but in the absence of oxygen no such movement took place. Stoppage of this movement in absence of oxygen was reversible.

Further studies on regeneration in Fundulus embryos. S. Milton Nabrit.

Due to the fact that time recorded in terms of days lapsed since fertilization is not an adequate criterion of developmental time for *Fundulus*, the results obtained from experimental development on this form cannot be readily compared. Some of the differences in results obtained after the removal of the distal end of the tail of the fish may be accounted for on that basis. Other differences, however, are not so readily explained.

The Nicholas [1] technique was employed to remove *Fundulus heteroclitus* embryos from their chorions six days after fertilization. At this time the embryos were fitted to the Oppenheimer [2] developmental schedule for normal development at 25° C. The embryos were between the stages 24–25, about 80 hours. The natatory fold was elevated from the distal end of the tail up to the third segment from the distal end. The rounding of the caudal fin had not begun. The operations for removal of the chorions and for the distal two segments of the tail were performed in amphibian Ringer's solution. After twelve hours some fish were transferred to sea water; after twenty-four hours some fish were placed in 4/5 Ringer's and 1/5 sea water by volume.

[1] Nicholas, J. S., 1927, *Proc. Nat. Acad. Sci.*, **13**.

[2] Oppenheimer, J., 1936, *J. E. Z.*, **73**.

About 20 per cent of the fish kept in the modified Ringer's solution and 10 per cent of those transferred to sea water differentiated tail fins without replacing the missing tail segments. New rays were first observed in those in the modified Ringer's solution in 7 to 9 days. New rays were observed in those in sea water in 11 to 16 days.

Birnie[3] reported that five-day-old fish cut in sea water or in isotonic sodium chloride solutions that afterwards were transferred to sea water did not differentiate tail fins in 65 days. I previously reported that cauterized tail stumps would differentiate new fins in fish that were 9 days old at the time of removal of the distal end.[4] Therefore, to assume that some of the fish in these experiments had not arrived at the critical period for setting the differentiation, and that most of them had, would necessitate the supposition that cautery causes a reversal or recovery of the capacity to differentiate the caudal fin. The other alternative appears to lie in the nature of the healing process. If sloughing occurs, the chances that the natatory fold will close over the cut stump in time to become infiltrated with mesenchyme are greatly diminished. In such cases rays may not differentiate. It is rather striking that if rays differentiate at all it is quite early. The fold is a regressive structure in *Fundulus* and appears to depend upon fin differentiation for persistence. I have not obtained caudal regeneration in the fry of *Fundulus*, although it has been reported in several other fishes.

The action of certain drugs on the intact heart of the compound ascidian, Perophora viridis. A. J. Waterman.

Previous work has been done on *Molgula*, *Ciona* and certain salps (Hunter, Schultze, Bacq, etc.). In *Perophora* the abvisceral beats (toward branchial basket) greatly outnumber the advisceral; both are highly variable.

Adrenalin, mecholyl and acetylcholine excite dominance of the advisceral center, but it is a question if they all act in the same way and on similar mechanisms. Adrenalin increases the number of abvisceral beats and the length of rest periods, decreases the advisceral beats and causes irregularity. In 1–125,000 dominance lasts a few minutes. In 1–20,000 the advisceral beats are suppressed for many hours when transferred during the abvisceral series; if during the advisceral, several reversals occur before the abvisceral become continuous ('death sign' of Schultze). After recovery in sea water or oxidation of adrenalin (*Perophora* accelerates the latter) these results are reproducible. Occasionally 1–100,000 increases the number proportionately in both directions. One to 15,000 inhibits dominance, and beating from both ends occurs without coordinating rhythm. Mecholyl (1–5,000 to 1–30,000) is less effective and abvisceral dominance lasts about 10–30 minutes. In acetylcholine the time varies from 4–9 minutes. Certain other effects are also different.

Atropine (1–2,500 to 1–5,000) causes irregularity, stops the heart in 5–30 minutes, and tends to reduce or abolish the exciting action of mecholyl; but the latter restores an atropine-poisoned heart to near normal. Acetylcholine and mecholyl influence the action of each other. Strychnine and colchicine are depressant without significant evidence of stimulation or of differential effect on the heart centers. Reaction and recovery occur quickly. No prolonged contraction of the animals occurred except with lethal concentrations of these drugs. Some of the observed effects differ from those described for other ascidians. In certain respects these results resemble those obtained with crustacean hearts.

[3] Birnie, J. H., 1934, *Biol. Bull.*, 66.

[4] Nabrit, S. M., 1938, *Jour. Exper. Zool.*, 79.

An effect of the injection of a solution of dihydroxyestrin into castrated female frogs, Rana pipiens. Opal Wolf.

In 1928 Wolf reported that subcutaneous injections over a long period of time, of a water-soluble extract prepared from mammalian ovaries prevented the involution of the oviducts of female frogs castrated in the autumn. Wolf also demonstrated in 1929 that implants of the anterior lobe of frog pituitary evoked the reproductive processes of both the male and female frogs, *Rana pipiens*, as far out of season as September.

Further studies in 1938 showed that the ovary and the oviduct of the frog during the summer months were enlarged following pituitary stimulation, the latter presumably as a result of an increased output of ovarian hormone.

The present study was undertaken to show the effect of injections of a solution in sesame oil of the pure crystals of dihydroxyestrin (a- estradiol benzoate).[1] Female frogs were castrated early in July when the oviducts are very small and were allowed to recover fully from the effects of the operation. Approximately five grams of lean beef were fed daily, the animals gained in weight and appeared in excellent condition. From August 10 to August 17 inclusive, a total of 1000 rat units per frog of the solution was injected into the thigh muscles. An average increase of 47.1 per cent in the weight of the oviducts in proportion to the body weight (more marked in diameter than in the length of the oviducts) of the injected animals over the castrate controls had occurred as a result of the eight days of injection.

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[1] The hormone was furnished through the courtesy of Dr. Max Gilbert of the Schering Corporation.

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THE EFFECTS OF A LACK OF OXYGEN AND OF LOW OXYGEN TENSIONS ON PARAMECIUM

J. A. KITCHING[1]

(*From the Department of Physiology, Princeton University, Princeton, N. J., and the Department of Zoölogy, University of Bristol, England*)

INTRODUCTION

The ability of *Paramecium* to survive without oxygen has been the subject of many investigations, and the results previously published are at variance with one another. Loeb and Hardesty (1895) confined *Paramecium* in a special gas chamber which they freed of oxygen either with a stream of hydrogen or by means of alkaline pyrogallol; the organisms died after twenty-four hours. Budgett (1898), using an Engelmann gas chamber flushed with hydrogen, obtained a survival time of several hours, after which the *Paramecium* blistered. Pütter (1905) found *Paramecium caudatum* to survive for five or six days in a specially designed bottle which had been flushed thoroughly with nitrogen. Fauré-Fremiet et al. (1929) got a survival time of twenty-four hours at 25° C. in sealed tubes containing leuco-methylene blue (reduced by sodium hydrosulphite). Juday (1909) discovered *Paramecium* sp. in the deeper waters of Lake Mendota (Wisconsin) at a time when he believed these waters to be devoid of free oxygen; according to him *Paramecium* is therefore able to live anaerobically for several months. Fortner (1924) enclosed *P. caudatum* in an atmosphere of hydrogen together with aerobic bacteria to remove any remaining oxygen; they survived for several hours, and their contractile vacuoles continued to function, although at a reduced frequency. Gersch (1937) found that *Paramecium* died within 10 seconds in gas purified of its oxygen by means of alkaline pyrogallol. In the work which is described in this paper I have attempted to harmonize these discrepancies.

In addition it has been claimed by several investigators that the respiration of *Paramecium* is insensitive to cyanide (Lund, 1918; Gerard and Hyman, 1931; Shoup and Boykin, 1931). I have therefore made some observations to determine the capacity of *Paramecium* to carry on its normal activities, such as secretion by the contractile vacuoles and locomotion, in the presence of cyanide.

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[1] Fellow of the Rockefeller Foundation.

APPARATUS AND METHODS

Anaerobiosis in a Hanging Drop

In subjecting Protozoa to a lack of oxygen, it is necessary to ensure the following conditions:

(1) A genuine lack of oxygen. It is not possible to assure complete absence of oxygen molecules, but the oxygen content of the medium surrounding the organisms must be so small that the organisms could not possibly make any significant use of it for oxidative metabolism. Oxygen must not be generated by accompanying plant cells or bacteria.

(2) No extraneous contamination. For instance, there must be no harmful impurities in any gas used. For this reason the absorption of oxygen by alkaline pyrogallol is perhaps to be avoided.

(3) No harmful secondary effects. For instance, the flushing of the medium with an oxygen-free gas mixture must not result in a harmful shift of the hydrogen ion concentration.

The general method used was to pass oxygen-free gas past a hanging drop containing the organisms. For this purpose cylinder hydrogen, or cylinder nitrogen (water-pumped), was first bubbled through concentrated sulphuric acid, dilute sulphuric acid, dilute potassium hydroxide and distilled water. Next it was purified of its oxygen. In most experiments hydrogen was used, and was passed through an electrically heated quartz tube containing platinized asbestos. The quartz tube extended sufficiently far at each end beyond the heating coil to avoid any significant warming of the deKhotinsky cement seals. In some experiments, however, nitrogen was passed over hot copper in a special internally heated furnace described by Kendall (1931). This furnace consists of a Pyrex glass tube containing a cylinder of copper gauze of large surface area, which is heated from within by means of a coil of nichrome wire. In this furnace it is always possible to see the condition of the copper, and in the time required for one experiment only a small section of the copper, at the inflow end, became tarnished. Finally the gas was carried by pure lead tubing with seals of deKhotinsky cement through a closed glass wash tube with distilled water in it to the chamber containing the organisms.

The organisms were mounted in a hanging drop on a coverglass which was sealed with vaseline or a mixture of vaseline and paraffin wax to the chamber. The hanging drop was not allowed to touch the vaseline. The chamber itself consisted of a glass ring about 2 cm. in diameter and 1 cm. deep, closed underneath by a microscope slide to which it was sealed with deKhotinsky cement, and with glass inlet and outlet tubes. The upper edge of the ring was ground to support a coverglass, and the microscope slide which formed the base of the chamber fitted onto the mechanical stage of the microscope.

From the chamber the gas was carried by lead tubing to a light-proof box, where it was bubbled either through a simple water trap or through a suspension of marine luminous bacteria. These bacteria (*Achromobacter fischeri*) luminesce in the presence of minute traces of oxygen, and according to Harvey and Morrison (1923) about 0.005 mm. of oxygen can be detected in this way. After the purified hydrogen or nitrogen had bubbled for five or ten minutes through the bacterial suspension no luminescence could be detected with the dark-adapted eye, but admission of air gave immediate recovery of luminescence. An analysis of the purified gas made by mass spectrograph by Dr. W. R. Guyer showed no trace of oxygen, although one part in 10^5 could have been detected.

For control experiments another glass chamber, similar to the first, was mounted on the stage of a second microscope, and CO_2-free air was drawn through gently with a suction pump.

Anaerobiosis in a Tube with Reduced Redox Indicator

The survival of organisms in the presence of a reduced redox indicator low in the redox scale has been used as a demonstration of anaerobic life. For instance, Clark (1924) found that certain bacteria reduced indigo carmine until a high ratio of the reduced to the oxidized substance was attained. He calculated that the oxygen tension in equilibrium with this mixture was so low as to be physically meaningless. For the present purpose, however, such a condition does not establish anaerobiosis, since the bacteria were obtaining oxidative energy by reducing the dye. The dye should therefore be reduced as completely as possible to avoid this possibility. Furthermore, the use of methylene blue, indophenols, or other indicators high in the series is to be avoided. The reoxidation of indicators high in the series is relatively slow at atmospheric oxygen tension (Barron, 1932), and might be very slow at low oxygen tensions even at the experimental hydrogen ion concentration. Harvey (1929) has shown that indophenols in the presence of luminous bacteria may remain reduced in the presence of a concentration of free oxygen sufficient to allow the bacteria to luminesce. Under certain conditions equilibrium may never be attained, and the state of the dye is then no indication of the oxygen tension in the solution. The method described below to the best of my knowledge avoids these errors.

Paramecium in the requisite medium was placed in an internally sealed glass wash tube with some platinized pumice and sufficient indigotrisulphonate to color the solution a clear blue. (Platinized pumice was found more satisfactory than platinized asbestos, as the latter is easily stirred up, and splits into fine sharp spikes which may damage the organisms. Platinized pumice was prepared by boiling pumice chips in chloroplatinic acid until most of the air had been driven out of the pores. Then sodium formate was added until platinum was precipitated in the pores and all over the surface of the pumice. The chips were then washed very thoroughly in running water.) The wash tube was sealed with deKhotinsky cement to the source of purified hydrogen, and the gas escaping from it was carried by lead tubing to the anaerobic chamber for a parallel experiment. The indicator dye bleached five or ten minutes after the hydrogen was turned on. The wash tube was then wrapped completely and thoroughly in black cloth so as to exclude all light, and slow bubbling of the hydrogen was continued. This precaution was taken although no photosynthetic organisms were ever detected in the cultures.

Controlled Oxygen Tensions

In order to subject *Paramecium* to controlled and known oxygen tensions, the apparatus used for anaerobiosis in a hanging drop was modified. Hydrogen, purified of its oxygen as already described, and oxygen were led through flow meters (see Harvey and Morrison, 1923) to a T tap in which they were mixed. The resulting known gas mixture was then conveyed through the wash tube with distilled water to the observation chamber. Lead tubing and seals of deKhotinsky cement were used throughout. According to a later refinement, condensation of water in the capillaries of the flow meters was prevented by a tubular show-case lamp placed alongside them. By manipulation of the T tap the organism could be subjected rapidly either to pure hydrogen or to any desired ratio of hydrogen and oxygen.

In a few experiments at very low oxygen tensions, oxygen was mixed with hydrogen in proportions measured by flow meters, and a small part of the resulting mixture was then mixed in the same way with additional pure hydrogen. The unwanted part of the first mixture was rejected through a mercury trap. In this way extremely low tensions of oxygen could be provided with reasonable accuracy.

The tension of oxygen in the observation chamber was calculated with due regard for the barometric and water vapor pressures. The total gas pressure in the observation chamber exceeded that of the air by an insignificant amount.

The Diffusion of Oxygen in a Hanging Drop

I am indebted to Dr. H. P. Robertson of the Physics Department, Princeton University, for a mathematical expression relating the thickness of the hanging drop, the original concentration of oxygen in it, the diffusion coefficient of oxygen in water, and the time required to bring that drop to any given low oxygen tension after the drop has been placed over an atmosphere devoid of oxygen. The drop has been regarded as a film parallel to the coverglass, which for my experiments is reasonably true. Of the infinite series which was derived only the first term is significant, viz.

$$t \sim -\frac{4b^2}{\Delta\pi^2} \cdot \log_e \frac{\theta\pi^2}{8},$$

where t = the time required for the equilibration,
b = the thickness of the drop,
θ = the ratio of concentration of oxygen attained at time t to initial concentration,
Δ = the diffusion coefficient of oxygen in water.

For conditions approaching those of the experiments Δ is given by Bruins (1929) as very nearly 2.0×10^{-5}.

It will be seen that the time required for the drop to reach any given oxygen tension varies as the square of its thickness. Let us choose arbitrarily an oxygen tension of $10^{-4} \times$ the oxygen tension of the atmosphere—less than one-tenth the minimal value necessary (as will be shown later) to allow activity in *Paramecium*. For $\theta = 10^{-4}$, when $b = 1/4$ mm., $t = 2$ minutes; and when $b = 1/2$ mm., $t = 8$ minutes. The films used were of this order of thickness, and in practice it is probable that equilibration is accelerated by the water currents set up by the swimming of the *Paramecium,* as well as by convection currents.

These theoretical results agree well with experiments on certain marine amoebae, to be published later, in which oxygen tensions below ½ mm. are required for stoppage of movement, and which in a hanging drop come to a standstill within five minutes of the time when pure hydrogen is turned on. It may be concluded that in my experiments adequately anaerobic conditions were attained within ten minutes. Similarly the equilibration of a hanging drop with gas mixtures of known low oxygen tension must be rapid, provided always that the drop is a thin one.

MATERIAL

Paramecium was cultured in timothy hay infusion, and was obtained from the following sources:

(1) *P. multimicronucleatum* collected from a backwater of the canal near Princeton, N. J. Individuals of this race had three contractile vacuoles.
(2) *P. multimicronucleatum* of a pure line (Clone I), with two contractile vacuoles.
(3) *P. caudatum* of a pure line (Clone D).

TABLE I

Survival of *Paramecium multimicronucleatum* clone I under anoxic conditions in a hanging drop of its own culture fluid. CO_2, when used, was at a partial pressure of 12 mm., which was sufficient to maintain the culture fluid at pH 7.1 approximately. Room temperature was 24–28°C.

Gas used	Time for first *Paramecium* to blister	Time for fifty per cent to blister	Time for last to blister	Number of animals used	Date (1939)
	minutes	*minutes*	*minutes*		
Pure H_2	160	370	440	14	August 6
Pure N_2	210	465	585	43	August 3
$H_2 + CO_2$	360	650	690	19	July 29
$H_2 + CO_2$	595	665	710	28	August 1
$N_2 + CO_2$	500	660	720	12	August 5
$N_2 + CO_2$*	500	660	725	9	August 7
CO_2-free air (control experiment)	—†	—	—	21	July 30–31

* Extra pure CO_2: see text.

† All survived throughout experiment (31 hours) and remained normal in appearance and activity.

The last two were both kindly supplied to me through the courtesy of Professor S. O. Mast of the Johns Hopkins University.

RESULTS

In all cases and in all media *Paramecium multimicronucleatum* and *P. caudatum,* when mounted in a hanging drop in absence of oxygen, continued to swim and to evacuate fluid by the contractile vacuoles for a limited time. However, the speed of swimming and the rate of output of the contractile vacuoles decreased, and eventually the organisms stopped. Then the trichocysts were discharged; the anterior end of the body usually, but not always, became constricted; blisters formed at the surface of the body; and cytolysis ensued. *Paramecium* which had ceased all activity for lack of oxygen, and which had even begun to blister, recovered rapidly on admission of sufficient oxygen (see Tables III and IV).

The duration of anaerobic survival was very variable, and depended partly on the media used. The longest survival times were got with culture fluid. In hanging drops of culture fluid survival times in absence of oxygen ranged from one to twelve hours. It appeared (as already shown by Pütter (1905)) that *Paramecium* from well-fed, flourishing cultures survived the longest. There were considerable differences between cultures of the same clone. However, exposure of a hanging drop of culture medium to pure hydrogen or nitrogen leads to a loss of carbon dioxide, with a resulting shift of the hydrogen ion concentration far into the alkaline range. Accordingly, pure hydrogen was bubbled through some culture fluid and the resulting shift of pH, according to measurements kindly made for me with the glass electrode by Dr. Marshall E. Smith, was from about 6.2–6.4 to about 8.9–9.0. Addition of about 12 mm. partial pressure of carbon dioxide to the hydrogen was sufficient to maintain the culture fluid at about pH 7.1. When hydrogen or nitrogen together with this amount of carbon dioxide were passed through the observation chamber, the *Paramecium* survived slightly but significantly longer than without the carbon dioxide (Table I); the series of changes leading to death was, however,

TABLE II

The effect of a lack of oxygen on *Paramecium caudatum* clone D, after segregation in test tube in culture fluid without hay.

Medium	Culture fluid; pure hydrogen	M/300 phosphate buffer; pure H_2	M/300 phosphate buffer; CO_2-free air as control
Experiments on same day:			
Time required for first one to stop or blister, in minutes	88	23	no visible adverse effects
Same for 50 per cent	154	56	—
Same for last one	235	76	—
Number of individuals used	28	8	12
Experiments five days later:			
Time required for first one to stop or blister	79	7	27
Same for 50 per cent	79	7	29
Same for last one	109	9	68
Number of individuals used	8	4	7

the same. In these experiments no purification of the carbon dioxide was undertaken, but the connection bewteen the carbon dioxide cylinder and the apparatus was entirely of glass, lead tubing, and deKhotinsky cement, and the pressure was regulated by a double water trap of such a nature as to prevent backward diffusion of oxygen. The manufacturers of the carbon dioxide stated that the oxygen content of their cylinders varied between two and sixteen parts in ten thousand. The oxygen content of the mixed carbon dioxide and nitrogen or hydrogen must have been insignificantly low, and experiments with a cylinder which had been cleaned out and filled with special care by the manufacturers gave entirely similar results.

TABLE III

The effect of distilled water, as compared with culture fluid, on the ability of *Paramecium multimicronucleatum* to withstand a lack of oxygen. For each gas mixture two separate hanging drops, with the two media, were suspended from the same coverglass.

Time	Pure Hydrogen		CO$_2$-free Air (Control)	
	Culture fluid	Distilled water	Culture fluid	Distilled water
minutes				
0	started	started	started	started
57	all very slow	1 blistered, rest very slow	normal movement	normal movement
64	1 blistered, rest very slow	"	"	"
73	all stopped	all stopped	"	"
74	3.5 mm. oxygen admitted			
80	3 swimming, rest appear dead	3 swimming. rest appear dead	"	"
395	2 swimming normally, rest dead	2 swimming normally, rest dead	"	"
Number of organisms used	15	12	12	8

The results obtained in the presence of a reduced redox indicator (indigo trisulphonate) in a wash tube in the dark agreed well with those obtained by the hanging drop method. In several experiments the purified hydrogen was bubbled first through the wash tube with the platinized pumice, and then through the anaerobic chamber. In all such experiments a few individuals survived in the wash tube after all had cytolysed in the anaerobic chamber. However, the wash tube contained many thousands of individuals, and it is to be expected that out of so many a few would be more hardy. At the time when the majority in the anaerobic chamber cytolysed there was a marked decrease in the number visible in the wash tube. The longest survival in culture fluid was about twelve hours.

In other experiments the organisms were washed four times with M/300 phosphate buffer ($Na_2HPO_4 + KH_2PO_4$) at pH 7.0–7.1, and mounted over the anaerobic chamber in this medium. Under anoxic conditions *Paramecium* underwent the same series of changes as in culture medium, although the constriction of the anterior end and the blistering seemed more sudden and violent. Also it survived for much less time in phosphate buffer than in culture fluid; even though in air it lived without apparent damage for many hours (in some cases observations were extended over two days) in the buffer solution. However, if some *Paramecium* were removed from the culture and placed in a test tube with some of the culture fluid but without hay, their ability to survive anaerobically in either medium (culture or buffer solution) decreased progressively, and after a few days they became fatally susceptible to phosphate buffer even when in air. These results are illustrated in Table II.

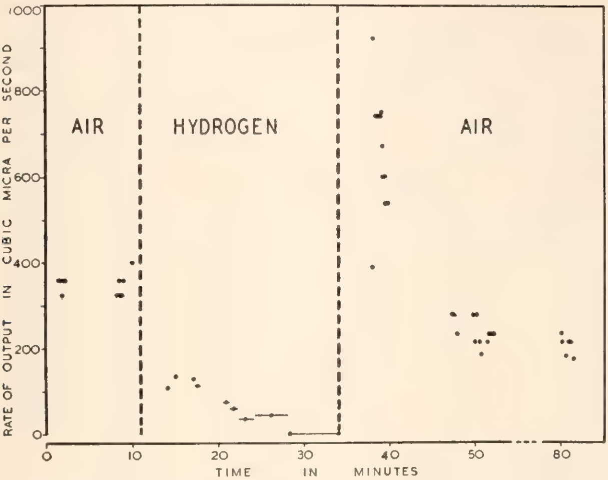

FIG. 1. The effect of pure hydrogen on the rate of output of the anterior contractile vacuole of *Paramecium multimicronucleatum* in a hanging drop of dilute phosphate buffer.

In the absence of oxygen *Paramecium* was found (in a few experiments) to survive equally well either in culture fluid or in distilled water (Table III). However, in M/300 KCl some individuals cytolysed almost instantly in air, and in absence of oxygen the remainder survived only a few minutes.

The contractile vacuoles, both in culture medium and in phosphate buffer, continued to function under anaerobic conditions, but finally became very slow. In two experiments *Paramecium* was slowed down by means of an agar gel just sufficiently viscous to make observation possible. The result of one of these experiments is plotted in Fig. 1. The stoppage of the contractile vacuoles after a period of lack of oxygen was found to be reversible. Contrary to the findings of Frisch (1937),

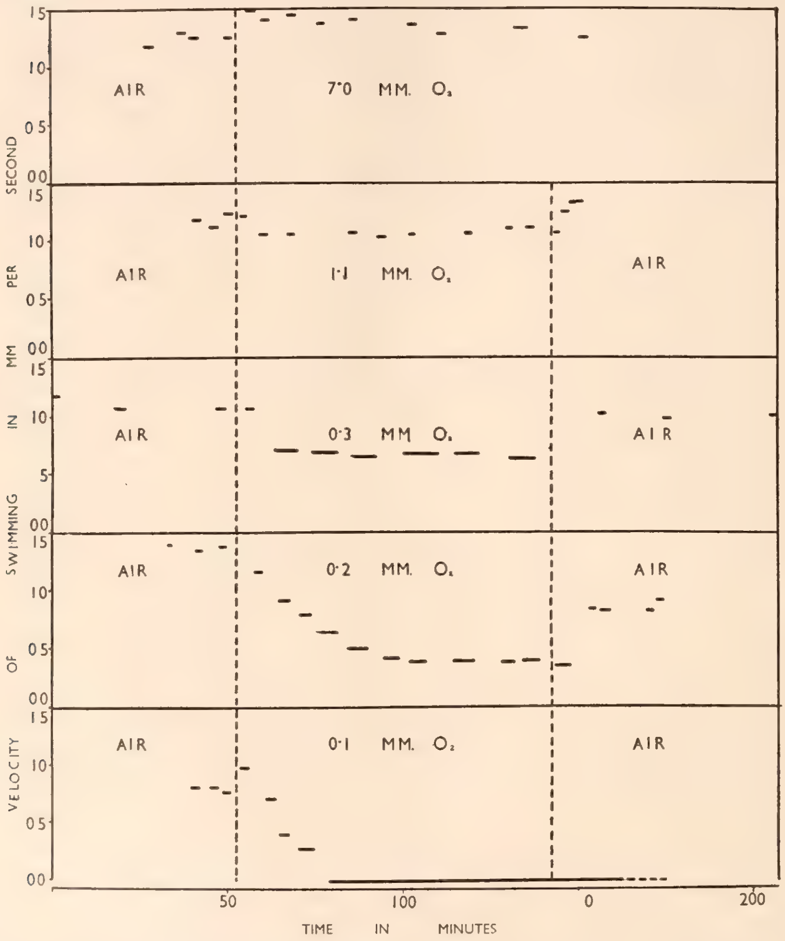

FIG. 2. The influence of low oxygen tensions on the rate of swimming of *Paramecium multimicronucleatum* in dilute phosphate buffer.

the *Paramecium* which I used showed normal activity of the contractile vacuoles whether they were swimming or stationary. This was true of *Paramecium* in hanging drops of culture medium or dilute buffer in contact with air, with or (usually) without agar. I attribute the discrepancy to the fact that his organisms had been sealed in a vaseline ring for many days and were not fully active.

A series of experiments was made to determine the survival and activity of *Paramecium multimicronucleatum* at low oxygen tensions. The rate of swimming of healthy *Paramecium* in phosphate buffer was found, after an initial burst of high activity, to remain reasonably constant for as long as observations were continued (up to 22 hours).

TABLE IV

Recovery of *Paramecium caudatum* at known oxygen tensions from lack of oxygen, in dilute phosphate buffer solution.

Duration of lack of oxygen in minutes	Condition at end of anaerobic period: number of animals		Oxygen admitted in mm.	Duration of observations at this tension in minutes	Condition after admission of oxygen at this tension: number of animals		
	Stopped but normal in shape	Stopped but pointed and blistered			Recovered after stoppage	Recovered after stoppage and blisters	Cytolysed
44	1	2	27	36	1	1	1
46	6	2	7.0	38	6	1	1
31	5	2	0.28	21	3	1	3
36	1	7	1.4	38	1	6	1
31	1	5	0.85	41	1	1	4
57	3	2	0.57	22	3	0	2
53	11	4	0.23	24	1*	0	14
36	3	1	0.17	18	0	0	4

* Movement very slow.

Accordingly single organisms were acclimatized for 90 minutes in a hanging drop of the buffer solution in a stream of moist carbon dioxide-free air. After this observations were made of the rate of swimming (*a*) in carbon dioxide-free air, (*b*) in a known mixture of oxygen and hydrogen, and (*c*) in carbon dioxide-free air. The second period (*b*) lasted usually about two hours. Readings of the time required for the organisms to traverse the distance indicated by the ocular scale (2.06 mm.) were made in groups of twelve, and the mean for each group determined. The standard error of the mean time usually lay between 3 and 5 per cent of the mean value. Results are shown in Fig. 2. There was a slowing down of swimming in oxygen tensions below 1 mm. Below 0.2 mm. approximately the organisms died, but above this value they slowed down to a speed approximately constant within the duration of the treatment; and this effect was reversible. Individual variation was such that it did not seem worthwhile to try to determine a detailed relation between oxygen tension and rate of swimming.

In a further series of experiments a group of *Paramecium* was mounted in a hanging drop of the dilute phosphate buffer in the anaerobic chamber, and subjected to anoxic conditions until all had stopped and some of them had blistered. Then oxygen was admitted at a known partial pressure, and the extent of recovery recorded. Results are shown briefly in Table IV. Recovery could be obtained, on admission of sufficient oxygen, even after the organisms had begun to blister, and in such cases the blisters were gradually resorbed. In many of these cases it is certain that if the organisms had been left for another one or two minutes without oxygen irreversible cytolysis would have ensued. The minimal oxygen tension needed for recovery was of the same order as that which was found just to allow swimming in the previous series of experiments.

The effect of cyanide on the secretory activity of the contractile vacuoles and on the general activity of the animals was examined briefly in three experiments. Single individuals of *Paramecium multimicronucleatum* were mounted in a hanging film of river water just sufficiently thin to prevent too rapid swimming. The cover glass was sealed with vaseline over a small glass cell half filled with river water, so that evaporation from the film was prevented. After a period of examination in river water the *Paramecium* was transferred with the usual four washes to a dilute solution of sodium cyanide (M/200, M/1,000, M/2,000, pH corrected to 7.1) in river water, and the fluid in the cell was also replaced by the cyanide solution. This procedure was carried out as quickly as possible in order to avoid loss of cyanide, and the examination of the organism was continued. After an initial depression of activity the vacuoles continued to function regularly although slightly less vigorously than in plain river water. The cilia also continued to beat actively. Observations were continued in one case for over ten hours after the cyanide treatment was begun.

Discussion

The majority of workers are agreed that *Paramecium* at room temperatures (20–25° C.) can survive for some hours, though not days, without oxygen. In view of the indubitable demonstrations of this fact, the results of Gersch (1937), who observed almost instantaneous death, must be discounted. It seems somewhat questionable whether Pütter, who (1905) obtained a much longer supposedly anaerobic survival, really achieved strictly anoxic conditions; and it is clear that *Paramecium* can make some use of oxygen at partial pressures below 1 mm. It also seems doubtful whether Juday (1909) would have detected oxygen in such low concentrations in the bottom waters of Lake Mendota (Wisconsin), where he claimed that *Paramecium* lived anaerobically. However, it remains possible that *Paramecium* might be found to survive without oxygen for longer periods under experimental conditions if it were supplied with suitable food. Slight discrepancies between the results of other workers may be ascribed to variations in the excellence of the oxygen "lack," possibly to the use of several different species, and particularly to variations in the state of nutrition of the organisms. This latter condition was stressed by Pütter (1905), and probably accounts for the beneficial effect of stirring the culture.

There is clearly some adverse influence in phosphate buffer which a normal healthy *Paramecium* can withstand in air, but which successfully operates against a starved *Paramecium* in air or against a well-fed one in absence of oxygen. The constriction of the anterior end of the organism and the blistering just before death are probably the result of a violent contraction of the myonemes. They were found to occur even when the organism was in M/20 lactose solution, and it is therefore unlikely that they can be attributed to osmotic uptake of water by the organism. A somewhat similar effect has been seen in *Paramecium* subjected to an electric current (see Kalmus, 1931). The phosphate buffer, either directly or indirectly, hastens the time for the myoneme contraction. It seems probable that the harmful effect of this buffer is due to a lack of balance of ionic concentrations, although this matter has not yet been investigated in detail.

Whereas cyanide in very low concentration inhibits the action of the contractile vacuoles of peritrich ciliates (Kitching, 1936), it has no very marked effect on those of *Paramecium*. The prolonged anaerobic activity of *Paramecium* might account for this. However, according to the results of various workers, at least a considerable part of the respiration of *Paramecium* must be insensitive to cyanide. Gerard and Hyman (1931) found that substitution of phosphate buffer for a calcium-containing water approximately halved the rate of oxygen consumption, but addition of cyanide made no further difference. The persistence of vacuolar activity in the presence of cyanide harmonises with the view that the respiration of *Paramecium* is relatively insensitive to this substance.

Paramecium is able to continue swimming, or to recover from a lack of oxygen, at oxygen tensions down to 0.3 mm. However, it is possible that higher tensions may be needed for growth and prolonged survival.

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Amberson (1928), by a not very delicate method, obtained results which suggest that the respiration of *P. caudatum* is depressed slightly at tensions of oxygen below 50 mm.

A comparison of the results obtained with *Paramecium* and with other Protozoa will be made in another paper.

ACKNOWLEDGMENTS

I wish to thank Dr. E. N. Harvey for his guidance and criticism, as well as for the facilities of the physiological laboratory, Princeton University. The work was completed at the Marine Biological Laboratory, Woods Hole.

SUMMARY

1. *Paramecium multimicronucleatum* and *P. caudatum* were subjected to pure hydrogen, pure nitrogen, and known mixtures of hydrogen and oxygen, while in a thin hanging drop under microscopical observation.

2. In all cases there was a limited period of anaerobic survival, during which activity of swimming and of contractile vacuoles was gradually diminished. Finally the organisms stopped, blistered, and cytolysed.

3. Admission of sufficient oxygen, even after blistering had begun, gave recovery.

4. Survival under anoxic conditions was best in culture fluid maintained at a reasonable hydrogen ion concentration by the addition of small quantities of carbon dioxide to the hydrogen or nitrogen. Under these conditions some organisms survived as long as twelve hours.

5. *Paramecium* was extremely variable as regards the length of anaerobic survival in any one medium. This variability is ascribed to physiological condition and not to genetic factors.

6. Survival under anoxic conditions was much shorter in dilute phosphate buffer than in culture medium. This is tentatively ascribed to a lack of balance of ionic concentrations.

7. In phosphate buffer the rate of swimming was reduced at tensions of oxygen below 1 mm., and the organisms died within a short time at tensions below 0.2 mm. At tensions above 0.3 mm. some measure of recovery from lack of oxygen could be obtained.

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DARK ADAPTATION AND REVERSAL OF PHOTOTROPIC SIGN IN DINEUTES

J. E. G. RAYMONT

(From the Biological Laboratories, Harvard University)

Introduction

Animals which exhibit orientation and directed movement with respect to a light source are said to be positively or negatively phototropic, according as they move towards or away from the source. Under certain conditions, a change of phototropic sign may occur, e.g. an animal which normally moves towards the source will move from it. Among the more important factors which have been shown to produce such a reversal of phototropic sign are: difference in absolute light intensity, change of light intensity, temperature, pH, and some chemical substances. The possible effect on phototropic sign of the degree of dark adaptation, however, has been largely neglected. Crozier and Wolf (1928) demonstrated the effect of dark adaptation on the strength of phototropism in *Agriolimax* and more recently Wolter (1936) has reported that some specimens of *Carcinus maenas* show a change of phototropic sign with dark adaptation. Since Clark (1931, 1933) demonstrated the process of dark adaptation by means of phototropic reactions for the " whirligig beetle " (*Dineutes assimilis*), it was decided to use this beetle in testing for the possible effect of dark adaptation on the sign of phototropism.

Material and Methods

The animals used in these experiments were of the species *Dineutes horni*,[1] and were collected from Fresh Pond, Cambridge, Massachusetts. They were kept in an aquarium in a lighted room, and were fed on pieces of fresh meat and fish, floated on wood.

The eye in *Dineutes* is divided into dorsal and ventral parts on each side, and the phototropic responses were compared when the whole, and when only part of each eye was functional. Blinding was effected by blackening the surface of part of the eye with asphaltum varnish.

In each experiment the beetle was first light-adapted and was then left for the desired period in a covered vessel of water. Following this period in darkness the phototropic reaction was observed by allowing the animal to crawl on a dry, horizontal test-plate of ground glass. This was illuminated from one side by a diverging beam of light emanating from a slit (1½" × ¾") covered by a ground glass plate, behind which a shielded 100-watt lamp was set. The test plate was level with the bottom of the slit and rested on a dull black surface. A little light was diffusely reflected up from the surface of the test plate but the greater part of the light reached the insect directly from the horizontal beam. The light intensities at the two ends of the test plate were 1.8 and 0.6 f.c. respectively.[2] The path taken by the insect was followed with a pencil, and marked directly on the plate. This did not appear to disturb the beetle.

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[1] I am indebted to Mr. C. Parsons of Harvard University for the identification of this species.

Light adaptation was effected by placing the beetle, in water in a glass cell (2¾" × ¾") for 20 minutes, in the path of a beam of light from a 500-watt projection lamp. A strongly-reflecting surface at the "darker end" of the cell ensured fairly uniform lighting. Intensity of adapting light was 4,800 f.c.[2] Heating was avoided during the light adaptation period by interposing heat-absorbing filters.

At the beginning of each experiment, the beetle was placed by means of a piece of stiff paper at approximately the centre of the test plate. It was not headed precisely in any special direction, as earlier experiments had shown that the animal oriented immediately, irrespective of the direction in which it was placed.

Observations

With all eyes functional, a strong, positively phototropic response was invariably obtained, whether the animal was light- or dark-adapted.

When any two, or when only one of the four eyes[3] was functional, a negative response was obtained if the beetle were dark-adapted. If only one eye was blackened the animal was photopositive. In the great majority of experiments, either the two upper, or the two lower eyes were covered. This avoided any complications, such as possible circus movements (cf. Clark).

It was often impossible to carry out all the experiments, with the various combinations of eyes, on the same individual, but the behavior was sufficiently constant to combine the results obtained from different animals. (A very few individuals were found in which the phototropic reactions were irregular.)

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[2] Light intensities were measured with a Macbeth Illuminometer, using dense filters for the highest intensities.

[3] To obviate needless repetition, the upper and lower halves of the eyes are simply referred to in the results as upper and lower eyes.

The results of experiments with (*a*) two upper eyes only functional and (*b*) two lower eyes only functional were similar. The beetles were always positively phototropic when they were light-adapted. They were usually still positively phototropic after dark adaptation of less than two hours duration, but became negatively phototropic after dark adaptation of more than two hours duration. However, the actual period of dark adaptation necessary to bring about the change in phototropic sign showed considerable individual variation; in a very few cases, even one hour was sufficient.

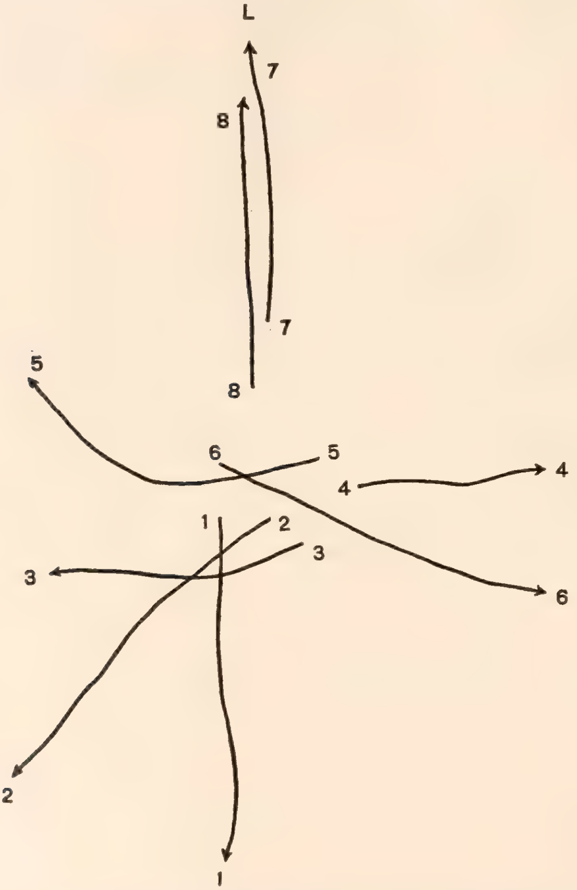

FIG. 1. Tracings of paths followed by *Dineutes* (Individual *X*) May 19, 1938, in 8 successive trials after dark adaptation for seven hours. The upper eyes were functional. The numbers refer to the order of trials. *L.* indicates light source.

A beetle which had been dark-adapted for a period sufficient to become negatively phototropic would show a reversal to the original positive condition as it became partially light-adapted. This was shown as follows. A beetle, after dark adaptation for many hours, was tested repeatedly in the beam. The responses exhibited at first were all photonegative, but the sign of phototropism became reversed after a certain amount of light adaptation had been brought about by the light from the test beam itself. Subsequently, all further responses were consistently photopositive.

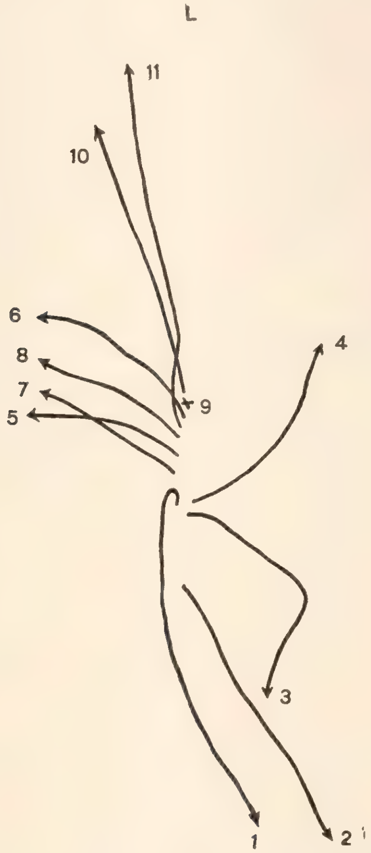

FIG. 2. Tracings of paths followed by *Dineutes* (Individual *B*) December 15, 1937, in 11 successive trials after dark adaptation for twelve hours. The upper eyes were functional. No " run " was obtained in Trial 9.

This reversal of phototropic sign with light adaptation was also observed, while avoiding the repetition of trials. A fully dark-adapted beetle was tested once to demonstrate the negatively phototropic reaction.

It was then left in the test beam, surrounded by a small glass cell, until the light effected a sufficient degree of light adaptation. A single new trial then showed the beetle to be positively phototropic.

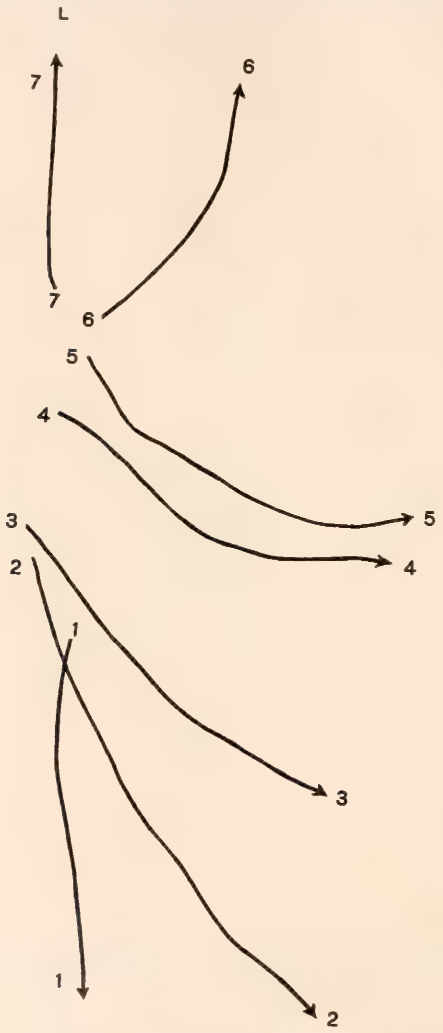

FIG. 3. Tracings of paths followed by *Dineutes* (Individual *V*) May 20, 1938, in 7 successive trials after dark adaptation for seven hours. The lower eyes were functional.

It was obvious that a considerable period of exposure to darkness was necessary to elicit the negatively phototropic reaction. Experiments were next carried out to test the possibility that with all eyes functional, a very prolonged period in darkness might cause a reversal of phototropic sign. The results of these experiments, with seven individuals, showed that reversal cannot be brought about by 24–65 hours of dark adaptation, if all eyes are functional.

If the two upper or the two lower eyes were blackened but a very few ommatidia of one of these eyes were left exposed, the beetle remained photopositive when fully dark-adapted. On completely covering the eye, the beetle, when dark-adapted, became negatively phototropic.

A few experiments were carried out with any three eyes blackened, and other experiments in which one upper eye and one lower eye of the opposite side were blackened. Such experiments, with any two or any three eyes covered, always showed that the dark-adapted beetles were negatively phototropic, and that light adaptation caused a reversal to positive phototropism.

TABLE I

Change in the direction of phototropic path followed by Dineutes (Individual V), in 7 successive trials, consequent upon light adaptation. The animal was first dark-adapted for 7 hours. The lower eyes were functional.

Trial Number	Angle of Deviation from Normal Positive Path
	degrees
1	180
2	150
3	135
4	110
5	110
6	50
7	0

Exact time of each trial, since beginning of exposure to test light was not recorded.

On the orthodox Loebian view, it would be expected that on repeatedly testing an originally fully dark-adapted beetle, a number of photonegative trials would be first obtained, and then, if a reversal of sign occurs with light adaptation, a sudden and complete change to positive phototropism would be observable. It was actually found, however, that an incompletely light-adapted beetle pursued a path *at an angle* to the light beam. If the direct negative response may be regarded as a deviation of 180° from the positive path, then with exposure to light, the angle of deviation gradually diminished until it approached 0° (i.e. the beetle was again positively phototropic).

It was possible to obtain such records from several individuals, and to repeat the observations on the same beetle. Although the actual paths (Figs. 1–3) were not straight, and in spite of some irregularities

TABLE II

Change in the direction of phototropic path followed by Dineutes (Individual B), in 6 successive trials, consequent upon light adaptation. The animal was first dark-adapted for 12 hours. The upper eyes were functional.

Trial Number	Total Time of Exposure to Test Beam	Angle of Deviation from Normal Positive Path
	minutes	*degrees*
1	0	180
2	1	120
3	3	75
4	8	60
5	18	(no "run" obtained)
6	38	20

in respect of angle, in general the results showed a surprisingly consistent trend.

The time required for complete reversal of phototropic sign varied from < 10 to > 30 minutes. It is believed that a rough estimate of the course of light adaptation can be obtained by measuring the successive deviation angles (Table I).

TABLE III

The effect of dark adaptation on the direction of phototropic path in Dineutes (Individual V). The lower eyes were functional.

Time in Dark Following Standard Light Adaptation	Result of Single Trial in Test Beam: Angle of Deviation from Normal Positive Path
minutes	*degrees*
5	5
15	35
24	90
30	(140)
45	120
90	160
180	180

Table II shows an experiment conducted on a beetle which was dark-adapted for a longer period. The total duration of exposure to light from the test beam since the beginning of the experiment was also recorded for each trial.

If these results do really indicate the course of light adaptation, it should be possible, using similar methods, to follow the course of dark adaptation. To test this possibility, a number of experiments were carried out using different periods of dark adaptation. In each experiment the animal was first light-adapted by means of the usual adapting light for 20 minutes. It was then dark-adapted for a given period, and a single trial made in the test beam. By using various periods of dark adaptation a number of trials were obtained, and the results (e.g. Table III) showed that with progressively longer periods of dark adaptation, the paths pursued showed an increasingly greater deviation from the positive path. An approximate dark-adaptation curve has been constructed from these data (Fig. 4) for one individual.

The possibility that the change in the paths pursued might be caused by the repeated disturbance of the beetle when replacing it at the centre of the test plate was investigated. The test plate was constantly moved in such a way that a beetle which had been originally fully dark-adapted was kept in the beam without being replaced. The animal at first moved from the light source, but with continued exposure it turned gradually towards the source until it reached the edge of the test plate. The changes in path are therefore not the result of disturbance, but do depend on the degree of adaptation.

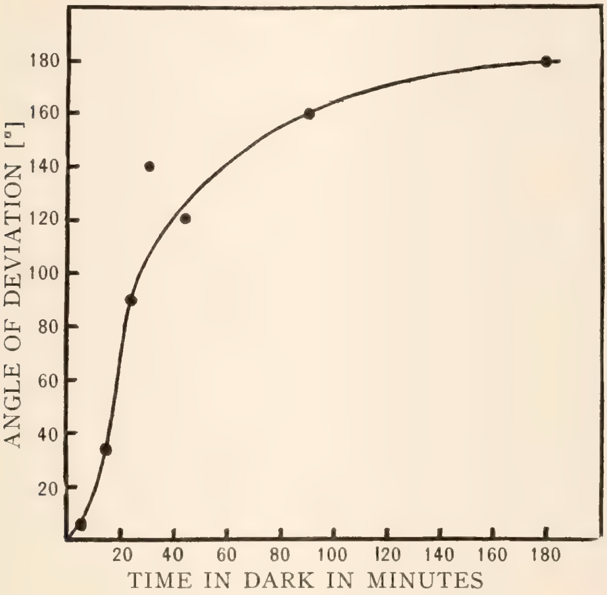

FIG. 4. Progressive deviation from the normal positively phototropic path during reversal of phototropic sign with dark adaptation, in *Dineutes horni* (Individual *V*). (The lower eyes were functional.)

DISCUSSION

McIndoo (1929) states that Schmitt-Auracher believed there was a relationship between the state of adaptation and pigment deposition in insect ocelli and the sign of phototaxis. In the present observations on *Dineutes,* a reversal of phototropic sign could never be obtained when all four eyes were functional, but if *any* two, or *any* three, eyes were blackened, the fully dark-adapted beetles were always negatively phototropic. The difference in behavior may depend upon the area of photosensitive surface stimulated. Although one cannot compare human, subjective sensations with animal tropisms, it is interesting that experiments on the intensity discrimination of the human eye have shown that the use of a small test field of high intensity may cause uncomfortable glare and even pain, while with a larger test field of the same average intensity, vision is normal.

When only partially light- (or dark-) adapted, *Dineutes,* with only two eyes functional, moves at an angle to the light beam. Rádl, Carpenter, and especially Dolley (1916) and Clark (1931 and 1933) have discussed movement of phototropic insects at an angle to a beam of light, but in all such cases only *one* of a pair of symmetrical eyes was functional, and deviation might be then expected. Clark supposed that light from the direct beam, and light from the background acted on the functional eye. But in the experiments described, with both upper, or both lower eyes functional, light from both background and beam should act equally on the two sides, and therefore, according to Loeb, the insect should move *directly* to or from the source. Indeed, Clark states that if in *Dineutes* both upper or both lower eyes are blackened, the beetle moves straight towards the light. But in Clark's experiments the beetles were consistently photopositive, and provided *D. horni* is strongly positively phototropic, it moves straight to the source also.

Mast (1938) has shown that the phototropic reflexes in insects vary according to the region of the eye stimulated. In *Dineutes,* according to Clark, the posterior ommatidia are much more sensitive than the anterior ommatidia. Possibly then, during reversal of phototropic sign with light adaptation in *D. horni,* some of the ommatidia become light-adapted more rapidly and give rise to reflexes which are opposed by the less sensitive ommatidia. Morphologically symmetrical ommatidia also may not adapt at exactly similar rates, and therefore photochemical reactions will proceed at different rates on the two sides during partial adaptation, and a deviation would result. When *all* ommatidia are fully adapted, the beetle will move straight to the source.[4]

Although an exact explanation must therefore await further work, it is obvious that the simple Loebian theory will not account for the facts here presented. Light must act in a more complex manner, and among other factors, the phototropic responses must depend to a considerable extent upon the region of eye stimulated, as Mast has repeatedly emphasized.

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[4] Some recent experiments on the related genus, *Gyrinus,* have shown that even with all eyes functional, a positively phototropic beetle, when it is fully dark-adapted, may pursue a path deviating widely from the normal straight photopositive path.

SUMMARY

1. When all eyes are functional, *Dineutes horni* is positively phototropic after dark or light adaptation.

2. With one or with two eyes functional, *Dineutes* is positively phototropic when light-adapted, but is negatively phototropic when fully dark-adapted.

3. At intermediate stages of dark and light adaptation, the beetle moves at an angle to the light rays. The courses of dark and of light adaptation were followed by a study of these "angles of deviation" from the incident rays.

4. Possible theories are discussed to account for these results.

ACKNOWLEDGMENTS

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MODIFIED SEXUAL PHOTOPERIODICITY IN COTTON-TAIL RABBITS[1]

THOMAS HUME BISSONNETTE AND ALBERT GEORGE CSECH

(From the Shade Swamp Sanctuary, Farmington, Connecticut)

INTRODUCTION

Studies too numerous to cite here show that the sexual cycles of many birds and mammals and of some fish and reptiles can be modified and their breeding seasons changed by manipulating daily cycles of exposure to light. Not all are susceptible in the same way nor to the same degree (Bissonnette, 1936, 1938; Marshall, 1936, 1937).

The development of proper methods of conservation and wild life management require knowledge as to what wild animals have photoperiodic breeding cycles and how they react to management of light-cycles. Breeding seasons of some animals have been prolonged to permit two litters per year in place of one, with better than normal growth of early-induced litters (Bissonnette and Csech, 1937, 1938, 1939).

The cotton-tail or gray rabbit of New England (*Sylvilagas transitionalis* (Bangs)) is shot for sport and food, furnishes food for fur-bearing carnivores, and may injure fruit trees in some places and seasons. It exhibits a limited breeding season with three or four litters, beginning about mid-April. It is, therefore, more polyoestrous than most of the animals so far investigated and intermediate between strictly monoestrous or dioestrous and completely polyoestrous forms. It, therefore, has been tested by " night-lighting " in autumn and winter.

MATERIAL AND METHODS

Three pairs of cotton-tails were placed, each in a wire enclosure raised from the ground, with wooden " den " at one end. In each den a few inches of earth were covered with loose dry grass about two inches deep for bedding. Twenty-five-watt bulbs were so placed as to shine into both den and enclosure and controlled by a time switch so that lights were lit for one hour each night for the first week, beginning on October 10. " Night-lighting " was increased one hour each ten days thereafter to eight hours on December 17 and maintained into February, and, in one case, into April. Lights came on each evening at six o'clock. All pens were outside, without heating, except from the bulb, throughout the experiment. Feeding and care were similar for all rabbits at the sanctuary.

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[1] Aided by grants from the National Research Council, Committee for Research in Problems of Sex, 1935–8, and from the Penrose Fund, American Philosophical Society, 1938–9, and by coöperation and animals from the State Department of Fish and Game, Connecticut.

Replacements were made without altering schedule when animals killed one another and the exact lighting history of each animal recorded. The gonads of killed animals were secured for histological study. But none were sacrificed expressly for such material, because our experience with raccoons and the behavior of these rabbits suggested that matings would lead to litters out of season. Sex-organs were obtained also from unlighted males on December 8 and January 25 for comparison with those of a male killed by his mate, January 12, after night-lighting eight hours each night from December 20.

Observations and Results

After varying periods of lighting and after matings, two experimental females killed their original mates by biting them through the back. One male killed his mate by grasping her anal region with his teeth and pulling out her abdominal organs which became useless for study. Replacement of males may account for failure of matings to induce pregnancy in December and again beginning on January 5. Controls also mated somewhat later in December and about January 10, all without pregnancies. Bissonnette's studies on male ferrets indicate that willingness to mate antedates potency and fertility by a considerable time. This was probably true here also.

After matings in January, two experimental females made nests and one lined hers with fur to receive young that failed to come. No control did so then, nor until near littering time in April.

The first "experimental" litter (of two) was born on April 4 and died April 10, from exposure. The nest was not heated and the mother left it for long periods. On May 31 and June 30 she produced second and third litters (of one and six) which survived. The first "control" litter came on April 18 and all six died of exposure. None of the other experimental or control females had litters before June 8, although the above-mentioned control female mated again the day her litter was born and others probably did so too. In that season no controls had litters live through. They normally should have had litters every thirty days after mid-April. It was a poor rabbit-breeding season, for reasons as yet unknown. Experimentally lighted animals succeeded slightly better than controls on normal light.

A male, used for replacement on December 20, lighted eight hours each night until killed by his mate on January 12, had mated about January 5–7 with her. His testes showed sperms just metamorphosed in small numbers in some tubules but none had yet reached the middle part of the epididymis. The apparent breeding condition of this epididymis, with tall columnar epithelium and well developed ciliary processes, indicates functional activity of the interstitial cells of the testis and accounts for sexual libido and matings.

Much smaller testes from the control of December 8 showed smaller tubules with no stages of germ-cells beyond synizesis. Its epididymis, in a partly activated, partly regressed condition, suggested some activity of interstitial cells, which may account for December and January matings of controls. Its epithelium was short columnar with some ciliary processes, less developed than those of the lighted male.

Testes from the January twenty-fifth control were slightly more advanced in spermatogenesis than those of December 8. Its most advanced stages were synizesis and a few growing primary spermatocytes. No germ-cell debris was found in the middle part of the epididymis which was more regressed than that of December 8. Its lining epithelium was more nearly cuboidal, with no ciliary processes evident.

Conclusions

Increasing night-lighting induces cotton-tail rabbits to undergo sexual activation in winter. In males, it leads to complete libido and spermatogenesis in twenty-three days at January 12, on eight hours of added light from December 20. It would probably induce complete breeding effectiveness in little longer time. In females, it induces repeated receptivity followed by nest-making and even lining of nests with fur.

December and January matings do not alone indicate increased sexual activation, for controls on normal light reacted similarly. Killing of mates is not attributable to added light; but rather it was permitted by the close confinement's preventing escape from an aggressor. Making and lining of nests in January, not done by controls at that time, signify activation above the normal for that time, and suggest pseudo-pregnancy after winter copulations.

Even if litters can be born under the conditions of temperature prevailing in these experiments in winter, they cannot be raised by their mothers, even in their fur-lined nests. The mothers do not keep the nests warm, as do raccoons, but leave them closed for long periods, returning only at intervals to suckle the young, born naked and defenseless against cold. It is suggested, however, that, with warmed nesting places, the long absences of the mother may not permit the young to die nor prevent the raising of litters in winter. In addition, by arranging runways so that males and females can have separate dens and can be separated after matings by a wire screen which permits them to remain acquainted without being able to kill each other, the same pairs may be kept on the lighting schedule and make winter breeding successful. Further experiments along these lines seem to be indicated.

Summary

1. Three pairs of cotton-tail rabbits were confined in dens and runways and subjected to increased lighting at night from October 10 onward. Controls were not lighted but fed similarly.

2. The original objective, induction of winter litters, was not attained because, after varying times of lighting, one member of the pair killed the other and replacements were made on schedule.

3. Sex organs of males were modified to complete sperm formation in twenty-three days in December and January and mating libido reached, accompanied by breeding conditions of the epididymis. Controls showed mating libido but no spermatogenesis nor epididymal activation.

4. Lighted females mated and made nests; and one lined its nest with fur. Controls mated but made no nests. No pregnancies resulted with any female until April.

5. The induced changes indicate that these rabbits can be brought into breeding condition in winter by increased lighting, but modification of the method used and the provision of warmed nest-boxes are necessary for successful winter breeding and rearing of these animals.

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THE LIFE CYCLE OF DACTYLOMETRA QUINQUECIRRHA, L. AGASSIZ IN THE CHESAPEAKE BAY[1]

ROBERT A. LITTLEFORD

(From the Chesapeake Biological Laboratory, Solomon's Island, Maryland)

Introduction

The common jellyfish or sea nettle of the Chesapeake Bay is the large scyphozoan medusa *Dactylometra quinquecirrha,* L. Agassiz. This species is a coastal form occurring in tropical and temperate seas throughout the world. In the Chesapeake Bay, as in other brackish water areas (Mayer, 1910; Menon, 1930), it is accompanied by a colorless, milky-white medusa which lacks the pigmented areas of *Dactylometra.*

The primary object of this study has been to elucidate the problems presented by the white medusa, which becomes sexually mature in a growth stage having twenty-four marginal tentacles, thus answering the taxonomic description of the genus *Chrysaora* of Eschscholtz. Certain workers, including Bigelow (1880), have considered the white medusa a member of the genus *Chrysaora,* on a basis of the sexually mature form, possessing twenty-four marginal tentacles. Mayer (1910) considers this medusa to be a growth stage of *Dactylometra quinquecirrha,* the so-called "Chrysaora-stage," believing that the premature development of the gonads is the result of the brackish water conditions. More recently Papenfuss (1936) has considered this white medusa to be a variety of *D. quinquecirrha* and named it *chesapeakeii.*

The literature on the life cycles of these two forms is limited to brief and scattered observations by Mayer (1910), Stiasny (1919–1921), Papenfuss (1934), and Truitt (1934). For this reason an investigation of the complete life cycles of the red and the white medusae was undertaken. The results obtained from this investigation are presented at this time.

Procedure

The organism was reared from the egg stage to the medusa under controlled conditions, and the data obtained in this way were supple-

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mented by observations in nature. Fertilization was obtained in the laboratory (1) by crowding sexually mature males and females together in a small container, and (2) by placing segments of the ripe gonads in fingerbowls 4 by 10 cm. in size and partly filled with brackish water.

The data reported in this paper are based on observations on between two and three thousand individual scyphostomae, about five hundred of which have been reared through four successive years. About one thousand additional embryos were followed to the scyphostoma stage.

The embryonic material was reared in a basement room of the laboratory, where changes in temperature and light were comparatively slight, though not controlled. The temperature of the water in the fingerbowls varied between 20 and 24° C. The lighting was indirect, light entering the room through a small window just below the ceiling on the eastern wall.

Scyphostomae were reared in the laboratory by placing a fingerbowl in a battery jar of three-liter capacity partly filled with bay water. Others were reared in fingerbowls placed overboard under natural conditions in nearby waters and protected by wire cages measuring $24 \times 9 \times 9''$. The wire was of hardware screen measuring eight strands to the inch. Each cage, containing five bowls held in a wooden rack, was lowered to the bottom in eight or ten feet of water in a protected cove near the laboratory.

The water containing the free developing embryos was changed daily by pipetting out a part of it and refilling the jars with fresh brackish water, whereas the water on the attached forms was changed weekly. The larvae were fed daily on material gathered by means of plankton tows, and on small pieces of oyster.

The embryonic material was studied by using hanging drops and depression slides, which made it possible to follow development of a single egg or group of eggs up to and through the planula stage. Material intended for cytological or histological examination was fixed in standard solutions of Kaiser's., Bouin's, Flemming's, or Zenker's fixatives and stained according to standard methods. Iron hematoxylin and Mallory's triple stain as given by Galigher proved to be particularly effective. Chloral-hydrate-menthol was most satisfactory for anesthetizing the scyphostomae.

OBSERVATIONS

Mature males and females of *D. quinquecirrha* may be recognized on a basis of the color of the gonads, which in the males are bright pink and in the females grayish brown or yellowish brown in appearance. The egg is a colorless cell with a prominent nucleus. The mature egg (Fig. 2), which is yellow in color, resulting from the filling of the cytoplasm with yolk material, varies in diameter from 0.07 to 0.19 mm., with an average diameter of 0.15 mm. The spermatozoa are developed in sacs (Fig. 1), many of which are found in a single gonad. These sacs show great variation in size and shape.

The eggs are released from the gonads into the stomach cavity and fertilization takes place either there or externally. Numerous laboratory observations strongly suggest that fertilization occurs in the stomach cavity. This is substantiated by the collection of fifty-one females bearing embryos in the gastric cavity.

The relationship between the initiation of development and the time of day is shown in Table I, which is based on 710 cases. These results are supported (1) by the collection, between eight and nine o'clock in the evening, of females having eggs in the first and second cleavage stages, and (2) by the collection before ten o'clock the next morning of females bearing gastrula and planula stages in the gastric cavity.

TABLE I

Time of Fertilization

Time	Number	Time	Number
3:00–6:00 p.m.	0	9:00–10:00 p.m.	206
6:00–7:00 p.m.	2	10:00–11:00 p.m.	24
7:00–8:00 p.m.	124	11:00–12:00 p.m.	16
8:00–9:00 p.m.	329	after 12:00 p.m.	9

The fertilized egg (Fig 3), which is distinguished by the presence of a distinct membrane, may divide immediately or it may remain quiescent for six or seven hours after fertilization. The first sign of development is the elongation of one end of the egg (Fig. 4), producing a prominent, knob-like protrusion (Fig. 5). This knob, the function of which is unknown, is resorbed prior to the first cleavage, which oc-

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EXPLANATION OF PLATE I

Photomicrographs from living material.

FIG. 1. Section of gonad of male medusa showing sacs containing spermatozoa. × 146.

FIG. 2. Section of gonad of female medusa showing mature and immature eggs. × 146.

FIG. 3. The fertilized egg. × 182.

FIG. 4. Elongation of the fertilized egg. × 182.

FIG. 5. Knob-like protrusion of fertilized egg. × 95.

FIG. 6. The two-celled embryo. × 146.

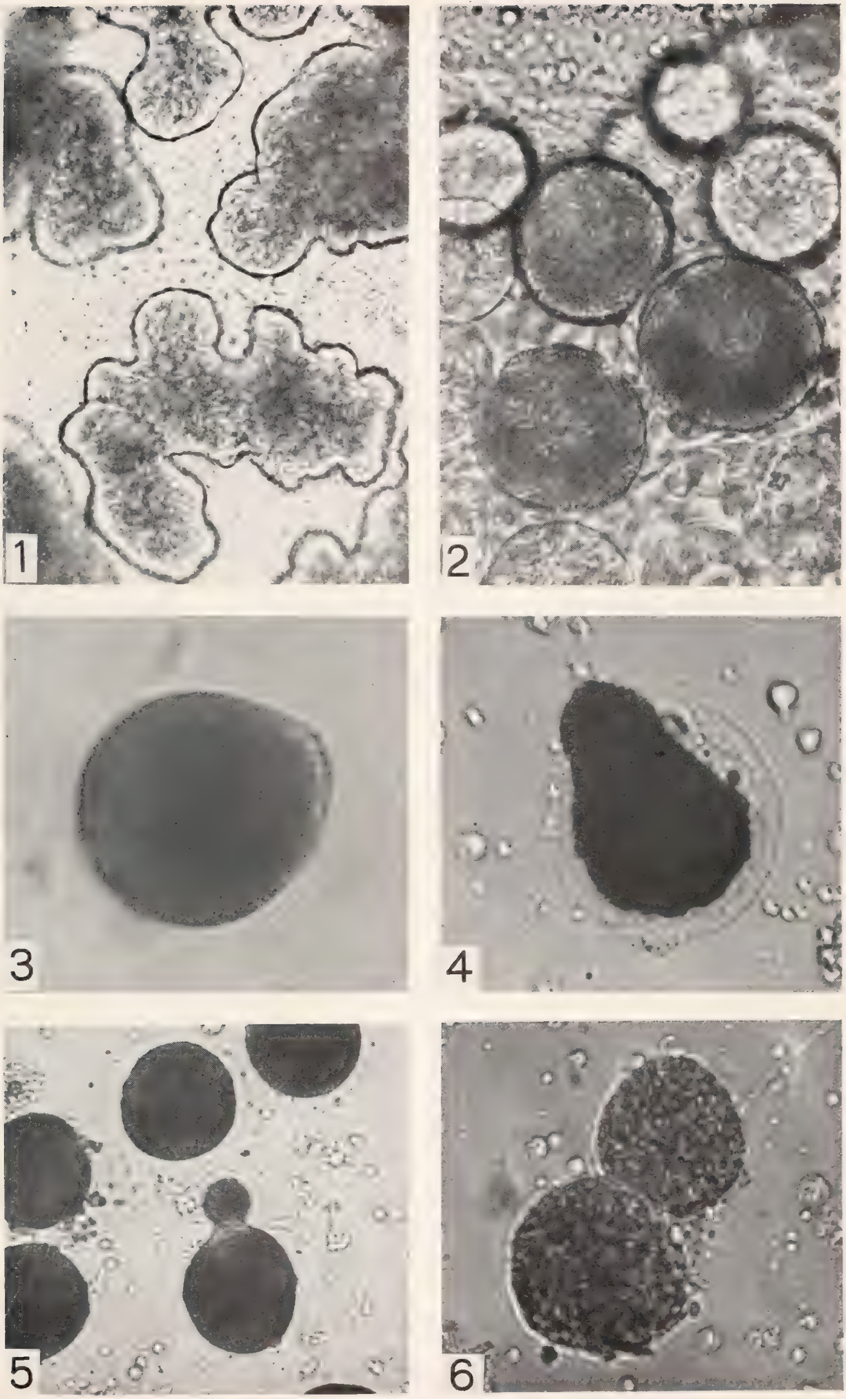


PLATE I

curs a little less than one hour after fertilization. The two-celled embryo (Fig 6) divides almost immediately to form four equal blastomeres (Fig. 7). At the end of three or four hours, under laboratory conditions, a third cleavage results in an embryo of eight equal cells. Succeeding divisions result in the formation of a blastula in about ten or twelve hours. During these cleavage stages the fertilization membrane, which surrounded the egg and embryo during early segmentation, is lost. The cells on one end of the blastula now invaginate and a free-swimming planula develops within sixteen to twenty hours after fertilization.

No provision is made for the protection of the developing embryo, such as the pockets on the oral arms of *Aurellia.* The rate of development of the embryonic stages shows great variation among individuals as well as with the external factors of the environment. A more detailed discussion of the stages of cleavage and gastrulation is to be presented in a subsequent paper.

The planula (Fig. 8) is at first round or oval in shape, but within two or three hours it adopts a definite pyriform outline. The first sign of movement in the planula is a regular rotation that is observed at the end of twenty or twenty-four hours. This is produced by the action of cilia which are developed by certain cells of the ectoderm. After adopting the pyriform outline, the planula moves through the water by means of a fringe of cilia confined to the cells of the periphery. Movement is rapid and the direction is changed continuously. The broad end is always directed anteriorly.

THE SCYPHOSTOMA

Completing its free-swimming existence after a period varying from three to five days, the planula becomes attached to some object, and is then known as the scyphostoma or hydra-tuba. The larva thus formed has a " ninepin " shape. In the process of attachment, the cells of the anterior region secrete an adhesive substance which forms a disc surrounding the stalk of the polyp.

The scyphostoma stage of *D. quinquecirrha* follows the general pattern of the group and lends support to the statement of Fowler (1900)

EXPLANATION OF PLATE II

Photomicrographs from living material.

FIG. 7. The four-celled embryo. × 165.
FIG. 8. The planula previous to adopting a pyriform outline. × 165.
FIG. 9. Scyphostomae having three to seven tentacles. × 26.
FIG. 10. Schyphostoma having eight tentacles. × 66.

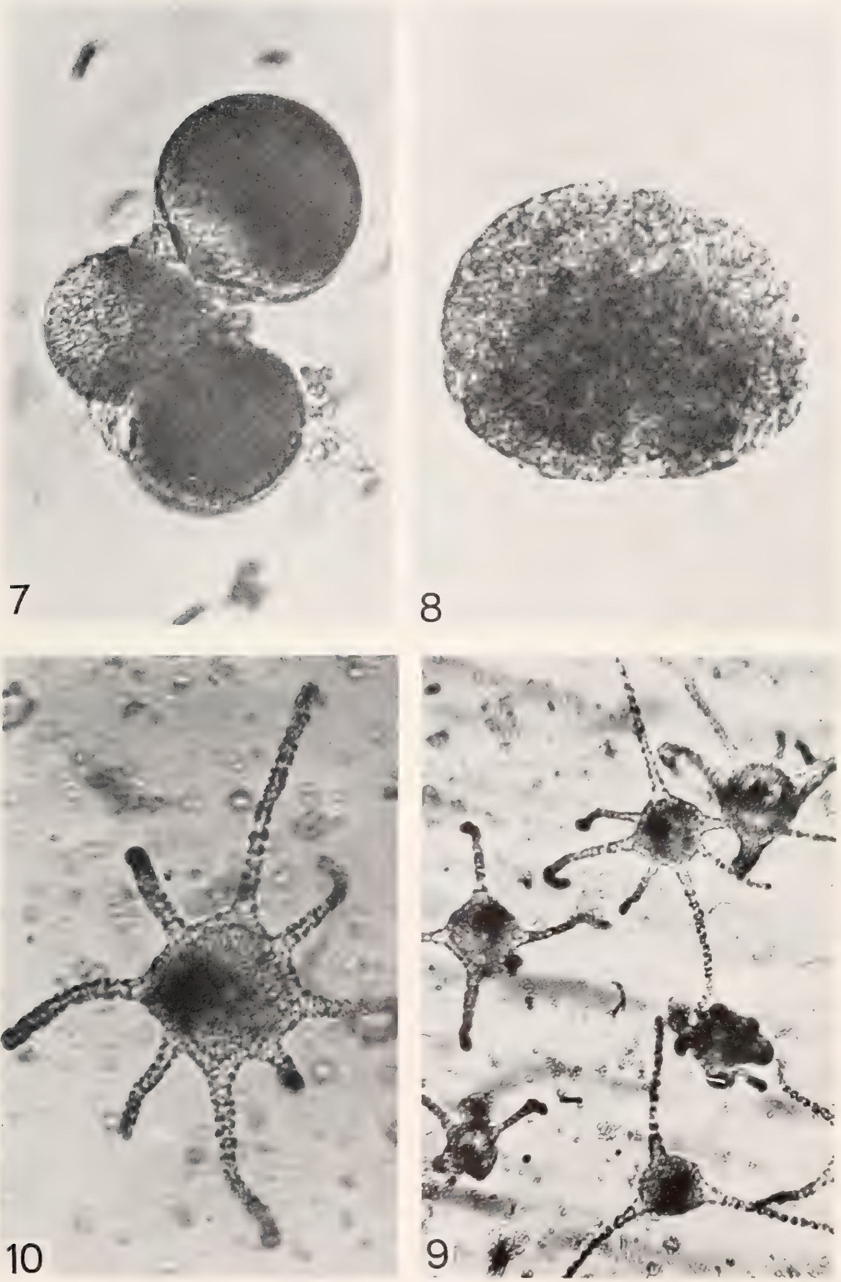


PLATE II

that the scyphozoan polyp is insignificant in size and monotonous in structure. The adult scyphostoma, which has sixteen or, rarely, twenty tentacles, shows three clearly defined stages of intermediate development, having four, eight, and twelve tentacles respectively. This fact probably explains the observations of Mayer (1910) that reproduction was through a scyphostoma stage having normally four tentacles, and of Stiasny (1919) that the number of tentacles was eight.

Development

Immediately after attachment, the oral region shows a great proliferation of endodermal cells resulting in the formation of the oral cone. This development, in turn, stretches the ectodermal layer and changes the mouth from a small almost invisible slit into a wide, yawning opening. This process is completed in from one to three hours after attachment. The sides of the oral cone become secondarily cleft to form a cruciform mouth.

The tentacles are produced singly and arise as wart-like evaginations from the region of the body surrounding the mouth. At the end of five days, the scyphostoma has four primary tentacles (Fig. 9) developed in the perradii of the body. Alternating with these tentacles, there arise four taeniolae, or ridges of the ectoderm, projecting into the stomach cavity in the interradial axes.

A circular depression appears on the upper part of the larva on the sixth day, marking off the oral cone from the bases of the tentacles. Several days later four deep depressions, the septal funnels, appear in this furrow just above the taeniolae. At the end of ten days a scyphostoma with eight tentacles (Fig. 10) is produced as the result of the formation of four secondary tentacles in the four remaining interradii. The scyphostoma continues to increase in size, and at the end of fifteen days, eight more tentacles have been developed in the adradii of the polyp (Fig. 11). Although the typical number of tentacles is sixteen, occasionally an individual which has developed twenty tentacles will be noted. This, however, is a rare exception.

EXPLANATION OF PLATE III

Photomicrographs from living material.

Fig. 11. Oral view of scyphostoma having sixteen tentacles. × 26.

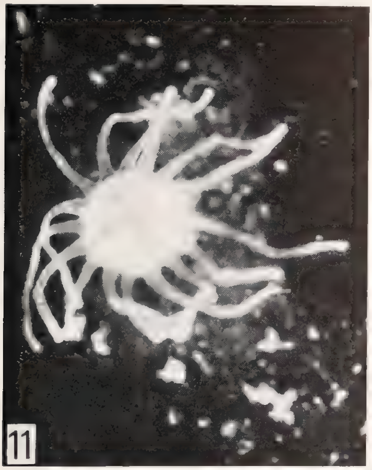
Fig. 12. Lateral view of scyphostoma showing the pedal discs. × 26.

Fig. 13. Colony of scyphostomae. × 10.

Fig. 14. Apical view of the strobila. × 26.

Fig. 15. The ephyra immediately after being released. × 46.

Fig. 16. Contracting ephyra showing the cruciform mouth, the heavy nematocyst aggregations, and the beginning of the gastric cirrha. × 119.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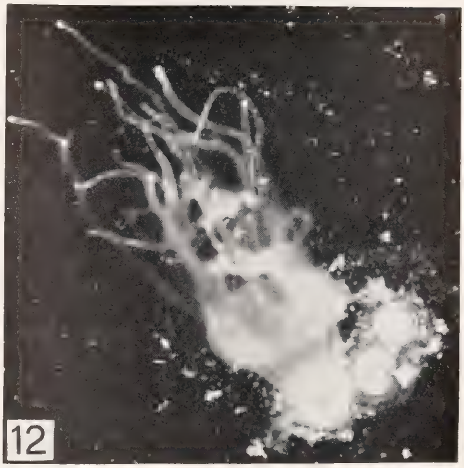
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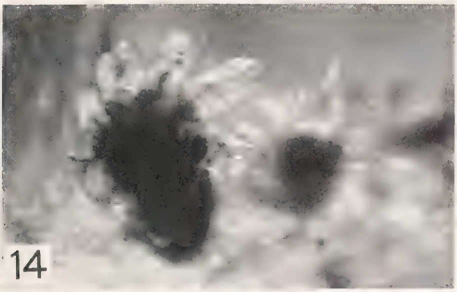
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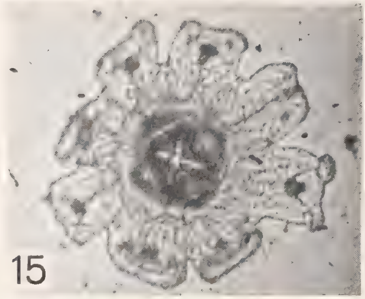
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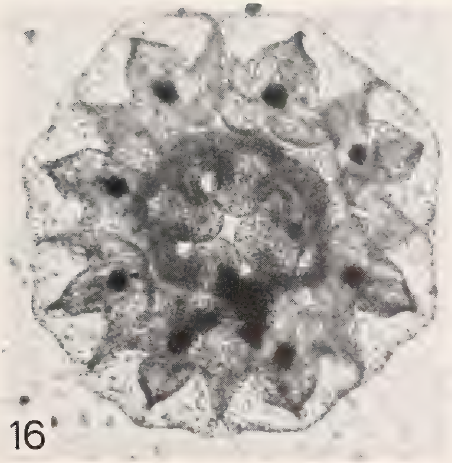
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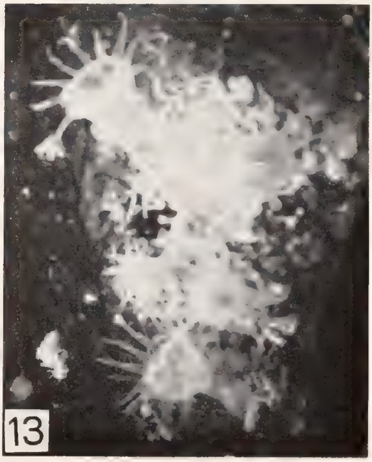
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PLATE III

In typical specimens, the scyphostoma has reached complete development by the middle of August, although longer periods are required for certain individuals. The only observed change taking place from September until the following April or May is a slight increase in size. The scyphostoma then becomes bright pink in color and begins to undergo a process of transverse fission known as strobilization.

After strobilization is completed, the remaining scyphostoma, which is about one millimeter in height, has a small cruciform oral slit, surrounded by sixteen normal-size tentacles produced before the ephyral discs were released. It may be distinguished from those scyphostomae which have not undergone strobilization by (1) the small mouth opening, (2) the short and broad appearance of the body, and (3) the crater-like mouth area in contrast to the normal oral cone. This old scyphostoma regains its normal appearance in from five to seven days. The following spring it again undergoes strobilization, producing the typical number of ephyrae.

Associated groups or colonies of scyphostomae (Fig. 13) are a regular occurrence in *D. quinquecirrha.* Such colonies, consisting of seven or eight individuals ranging in height from one to four millimeters, are produced by means of (1) stolons, (2) buds from the stalk of the parent scyphostoma, and (3) development of scyphostomae from the pedal discs. The pedal discs (Fig. 12) are formed as a result of movement of the scyphostoma from place to place, in the course of which it leaves behind on the substrate a protoplasmic disc. The groups of polyps developed from these discs may be either linear or irregular in formation, depending on the movement of the "parent" scyphostoma. The colonies formed either by means of buds from the stalk or by means of stolons are irregular in arrangement because of the irregular development of buds and stolons.

During the course of this study, it was observed that certain culture dishes in open water no longer contained scyphostomae. Instead, the bottom surface was covered with small, brownish, wart-like cysts. In one case (December, 1935) 51 cysts were counted and their positions marked on the culture dishes. The following February, 21 cysts had disintegrated, while normal scyphostomae had developed in the remaining 30 cases. In November, 1937, several culture dishes containing cysts were brought into the laboratory. The cysts in these dishes were kept under constant observation from that time. A count of 27 cysts was made in one dish, and two weeks later it was observed that the number was reduced to eighteen. Scyphostomae were produced from these cysts, and development took place in a normal manner.

It is to be noted that the cysts occurred in both cases in material that was being held in cages under conditions as nearly natural as possible. Scyphostomae which were retained in the laboratory for long periods without food or change in water resorbed themselves until they diminished to the size of one-week-old forms, but at no time did they encyst. At present, therefore, we can offer no valid explanation for the production of cysts. The same phenomenon has been observed in *Chrysaora* by Chuin (1930), who also has been unable to offer an adequate explanation. In *Chrysaora* these cysts produce a ciliated larva which swims about and then attaches itself to develop into a polyp. In *D. quinquecirrha* a larva was not produced, but a large number of these cysts produced new polyps in the same manner as has been reported for pedal discs.

Morphology

The scyphostoma of *D. quinquecirrha* is a colorless, goblet-shaped polyp averaging 3.5 mm. in height, with a diameter of 0.6 mm. The mouth is cruciform and occupies the entire oral surface of the organism. The body of the scyphostoma is divided into two distinct regions: (1) the long, stalk-like, tapering basal region, and (2) the cup-like body, or apical region. The relative size of the two parts is dependent upon the degree of contraction of the polyp. The tentacles are solid, averaging 6 mm. in length, and bear many nematocysts, which are regularly arranged, forming a series of successive rings around the tentacle. The aboral end of the stalk is fixed to a sub-strate and is surrounded at the point of attachment by a pedal disc. In older specimens the pedal disc may be one of a group, each of which marks a previous place of attachment. The body wall is divided into the three layers, ectoderm, endoderm, and mesoglea. The mesoglea is a thin, almost invisible lamella separating the two germ layers in the early development of the scyphostoma. This gelatinous layer increases after the eight-tentacle stage until it becomes a prominent structure of the mature polyp, containing muscle fibers and other cellular structures produced by the germ layers.

Reproduction

Reproduction in the scyphostoma stage is of two types: (1) budding, resulting in the production of additional polyps, and (2) transverse fission, or strobilization, resulting in the production of ephyrae.

Additional scyphostomae are produced as the result of three types of budding: (1) somatic buds from the stalk region of the polyp, (2) production of stolons, which may be considered a form of somatic budding, and (3) development from pedal discs.

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Stolon formation and somatic budding, the common methods of asexual reproduction in the polyp stage of the *Scyphomedusae,* have been found to be rare in this species. Likewise the statement of Truitt (1934), that the polyp buds profusely, has not been borne out by this study. The common method of asexual budding in *D. quinquecirrha* was found to be by means of pedal discs, the formation of which has been reported by Hérouard (1907) as occurring in *Taeniolhydra roscoffensis.* Other investigators (Mayer, 1910) have claimed that Hérouard was dealing with an abnormality of development of *Aurellia* This study has shown, however, that the formation of pedal discs is a normal occurrence in *D. quinquecirrha.*

Strobilization occurs in April or early May (June or July under controlled conditions). At this time the scyphostomae become bright pink in color and develop a series of circular furrows in the wall of the flask-like part of the body. As these furrows become deeper, the oral tentacles of the polyp are resorbed. This process may be completed before further development of the furrows occurs, or the tentacles may remain until the furrows mark off a series of well-defined discs. The furrows eventually divide the polyp into a series of saucer-like segments connected by a central cord and borne on a slender stem. Each of these saucers develops eight deep clefts, which in turn separate the periphery into eight lobes. Each lobe becomes cleft to form a bifurcation. At the apex of each bifurcation a deeply pigmented club, the future tentaculocyst of the medusa, is produced. The scyphostoma reaches the stage of a strobila (Fig. 14) in a period of four or five days and the saucers are then released into the water.

The number of discs produced remains remarkably constant at either five or six. The process of separation of the discs from the base or stalk region of the scyphostoma is completed in from ten to sixteen hours. Shortly before being released, the discs begin to pulsate and continue a characteristic movement of short, rapid pulsations until release is gained.

The Ephyra

The general structure of the ephyra was observed by Stiasny (1921), and Mayer (1910) had earlier published figures of the ephyra drawn by Brooks.

The newly liberated ephyra (Fig. 15) is about 0.84 mm. in diameter, measured from the distal end of one arm to the distal end of the opposite arm. The most prominent structure of its anatomy is the rhophalia, or tentaculocyst, of which there are eight, one in the bifurcation of each of the ephyral arms. Tentacles are lacking. The manubrium measures approximately 0.23 mm. in length and is flared in the radii, forming a cruciform structure. The nematocysts are grouped in capsules of three different sizes and also appear singly covering the exumbrellar surface. The ends of the ephyral arms appear knobbed as a result of nematocyst aggregations. The middle of each of the arms is marked by the presence of a pair of large nematocyst capsules, and the region where the arms join the disc is heavily covered with nematocysts.

The ephyra spends the first three or four days of its existence close to the bottom. During this time the subumbrellar surface is outward and the manubrium is carried in an upright position. A single tentacle is developed in each of the deep clefts that separate the ephyral arms. These tentacles, which appear four or five days after the ephyra has become separated from the scyphostoma, are the eight primary tentacles of the medusa, and the lobes of the arms are the primary lappets of the bell margin. Following the appearance of the eight primary tentacles, the ephyral disc grows outward, filling the clefts which separate the arms until it reaches the radius of the rhophalia. The margin then becomes cleft into a series of lappets, the number of which varies with the age, bell diameter, and stage of development of the individual.

When the eight tentacles have formed and the manubrium has increased in length until it is slightly longer than the bell diameter, the bell becomes inverted and the manubrium hangs downward from the center of the subumbrella. Inversion occurs between the sixth and the eighth day in typical specimens. The oral lips of the ephyra, which are heavily covered with nematocysts, are simple folds of the body wall, produced from the connecting tube of the strobila. As the ephyra develops into the medusa, these lips become folded and develop curtain-like margins on their internal edges.

Four hollow, tentacle-like outgrowths, which are the first gastric cirrha (Fig. 16), appear on the subumbrellar surface at the interradii of the manubrium three days after separation. They increase rapidly until as many as thirty-five or forty appear in each interradius. An ephyra 5 mm. in diameter has eight gastric cirrha in each interradius, while the 8-mm. one has ten, and the ephyra of 15 mm. has twenty-five. These cirrha increase in number as development in the medusa continues. Development in the ephyra is completed in from six days to two weeks. Upon obtaining the bell shape, it is considered as the early "post-ephyral stage" of the medusa.

SUMMARY AND CONCLUSIONS

Investigation of the life cycle of *Dactylometra quinquecirrha,* L. Agassiz in the Chesapeake Bay has been in progress since 1935. The results have been obtained (1) from observations made on the organism reared under controlled laboratory conditions and (2) from specimens reared under natural conditions. These observations have been supplemented by continuous investigation of the life cycle as it occurs under normal conditions.

This study has shown that the metagenetic cycle, previously reported by Agassiz and Mayer (1898), requires a period of from ten to twelve months for development from the egg to the medusa. The collection of scyphostomae and ephyrae from the waters of the Bay, as well as the successful rearing of the polyp stage under natural conditions over a period of four years substantiates the opinion of Cowles (1930) that the sea nettle breeds in the Bay. There is no evidence to support the contention that it breeds in the salter ocean water and migrates into the Bay. As was pointed out by Littleford and Truitt (1937), ephyrae have been collected in large numbers up deep creeks and inlets at times when they were not found in open Bay waters.

The common method of asexual reproduction in the scyphostoma stage is by means of pedal discs, budding being of rare occurrence. In the course of the study, it was noted that the scyphostoma had the capacity to encyst under certain environmental conditions. These cysts later produced polyps that continued development in the normal manner. Strobilization differs markedly from that known to occur in other species, in that the number of ephyral discs produced is constant at either five or six, at no time approaching the condition where a large and variable number is produced, as in *Aurellia aurita*.

The fact that the scyphostoma can live for very long periods of time was pointed out over one hundred years ago by Dalyell (1836), the discoverer of the polyp of *Aurellia*. It is of interest to note that this investigation established the fact that in *D. quinquecirrha* the scyphostoma can live for rather long periods of time. Certain individual polyps have been reared through four successive years and have undergone strobilization each summer during that time.

The ephyra, when small, lives close to the bottom and swims with the subumbrellar surface upward. After development of the manubrium and the eight primary marginal tentacles, the normal position is attained. Growth of the ephyra is a rapid process; the organism may increase ten times in size within a few days.

The life cycles of the red and white medusae are identical as regards rate of development and actual size of the morphological stages. The two "varieties" readily interbreed and the resulting cross shows no deviation from normal in its developmental history.

ACKNOWLEDGMENT

The writer wishes to express his appreciation to Professor R. V. Truitt, Director of the Chesapeake Biological Laboratory, for suggesting the problem and for his continuous advice and assistance during its prosecution; to Dr. C. L. Newcombe and Professor N. E. Phillips of the University of Maryland for helpful advice and suggestions; and to Professor W. L. Threlkeld, of the Virginia Polytechnic Institution, for his association with the investigation during the summer of 1935.

Particular thanks are due Professor E. A. Andrews, of the Johns Hopkins University, for a critical reading of the manuscript and for offering valuable suggestions.

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THE BLOCKING OF EXCYSTMENT REACTIONS OF COLPODA DUODENARIA BY ABSENCE OF OXYGEN[1]

MORDEN G. BROWN

(*From the School of Biological Sciences, Stanford University*[2])

The encysted state of a protozoön may be considered one of high stability in that little or no energy is required for its maintenance. Cells in this condition are not in any dynamic equilibrium of diverse reactions, but in a static state. This investigation is concerned with the fundamental problems of the nature of the changes from the dynamic to the static and from the static to the dynamic state as found in the encystment and excystment of protozoa.

The excystment process of the holotrichous ciliate, *Colpoda duodenaria,* is more than a reactivation of metabolic enzyme systems. The process involves a redifferentiation of protoplasmic structures, cilia, etc., along with special physical-chemical systems such as the contractile vacuole system and in addition involves the processes for escape from the cyst membranes.

Though like all ontogenetic processes, the excystment process is thus complex, it may prove amenable to analysis since the encysted organisms may be made very nearly uniform and will remain in a resting state with little or no change until reception of an excystment-inducing substance from their environment. The uniformity of the cyst preparation is obtainable since encystment as well as excystment depends on environmental conditions which may be controlled (Taylor and Strickland, 1938). A standardized, constant biological material may thus be made available for an extended series of experiments, and quantitative as well as qualitative results compared throughout the series.

The investigation into the nature of the physiological processes involved in excystment has been (1) by chemical analysis of substances which will induce the process (see Haagen-Smit and Thimann, 1938) and (2) by determination of the relations between the excystment time (the time elapsed between substitution of the excystment solution for the salt solution in which the organisms are kept and emergence from the cyst membranes) and controlled environmental factors such as concentration of the excystment solution, temperature, oxygen tension, and x-ray irradiation (see Taylor, Brown, and Strickland, 1936; Brown and Taylor, 1938; and Brown, 1938*a*). This report presents the experimental data obtained in the study of oxygen tension as a limiting factor in excystment together with a further analysis of the physical-chemical processes of excystment of *Colpoda.*

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[1] This study comprises part of the Ph.D. dissertation (Brown, 1938*a*) and has been briefly reported at the Richmond meetings of the A. A. A. S. (Brown, 1938*b*). Equipment used throughout this research was made available directly through the courtesy of Dr. C. V. Taylor and indirectly through a grant from the Rockefeller Foundation to Stanford University for Dr. Taylor's research in chemophysical biology.

[2] Now in the Department of Zoölogy, Washington University, St. Louis.

Experimental

The *Colpoda* used in these experiments were carefully cultured and selected as to interfission age, then encysted in grooves in cellophane. The cyst-cellophane preparation was then thoroughly washed and then kept in a continuously flowing, dilute, balanced salt solution. The technique of making this preparation was developed by Mr. Strickland (Taylor and Strickland, 1935) and the cysts used throughout this study were prepared by him.

The time for excystment was determined by counting the number of still encysted organisms (100 to 150 at start of each test) at intervals throughout the period of emergence from the cyst membranes. The geometric mean time was then evaluated by graphical methods as described by Brown and Taylor (1938). This geometric mean time which is equal to the median excystment time is referred to throughout this paper as excystment time.

In each experiment a series of concentrations of excystment solution, Difco yeast extract, was used. This enables one to separate the excystment processes into two periods: (1) that inversely proportional to the concentration of the excystment solution, and (2) that independent of the concentration of the excystment solution (Brown and Taylor, 1938).

The control of oxygen tension necessitated the design and construction of a special airtight excystment chamber through which gases of various composition could be passed. This chamber must be mounted on a mechanical microscope stage and fitted to a microscope of approximately 150 × magnification. The final design (Fig. 1) was the result of a long series of improvements of chambers and mechanical stages. The chamber will contain a set of six Columbia dishes which can be successively observed by rotation of the glass plate forming the floor of the chamber. The upper, stationary part is made from a large Petri dish cover which is ground into the plate. This Petri dish cover is drilled at four points, a large hole, shown in the figure through which the microscope objective projects, and three small ones, one for the inflow of the gas mixture, one for its outflow into a ⅛-inch tube about a foot long, and one, normally sealed and close to the gas outlet tube, through which twice concentrated excystment solutions, previously brought to equilibrium with the oxygen tension being tested, could be added to equal amounts of salt solution in the excystment dishes with a negligible admixture of air. The joint between the cover and floor and all joints about the objective have been sealed with paraffin oil throughout each experiment.

In the first series of experiments, the partial pressure of oxygen was reduced to $1/10$ that of air (from 150 mm. Hg to that of tank nitrogen, approximately 15 mm. Hg for the tank used). The control experiments were identical in all respects with those with reduced oxygen tension except that air was flowing through the excystment chamber instead of the tank nitrogen. No differences of any kind were found when the excystment under an oxygen tension $1/10$ that of air was compared with the controls. A typical experimental run and two controls are shown in Figure 2. These results are in agreement with studies of respiratory rate under low oxygen tension for the free-swimming stages of the ciliates *Paramecium* (Lund, 1918, and Amberson, 1928) and *Colpoda* (Adolph, 1929); and further indicate that in none of the preceding work involving measurement of excystment time in solutions in contact with air was oxygen ever a limiting factor.

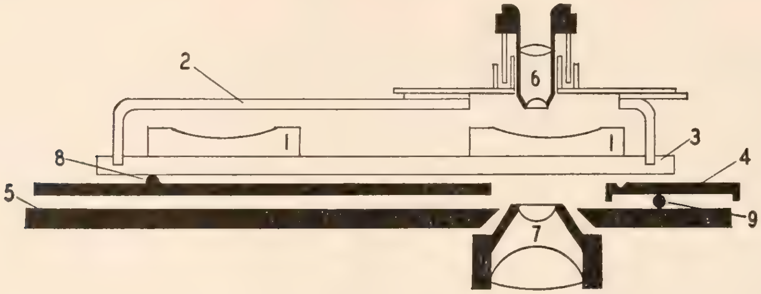

FIG. 1. Chamber and mechanical stage for studies of excystment of *Colpoda*. (1) Columbia dish containing cyst-cellophane preparation. (2) Glass cover of excystment chamber made from large, 150 mm., Petri dish. (3) Circular glass plate forming floor of chamber. (4) Moveable part of stage attached to a standard mechanical stage. (5) Microscope stage (specially constructed). (6) Microscope objective (10 × Zeiss, small size). (7) Microscope condenser. (8) One of three ball bearings in groove in moveable stage which support plate 3 and permit easy rotation of dishes. (9) One of three ball bearings between moveable stage and fixed stage.

To obtain lower oxygen tensions, the nitrogen was purified by bubbling through an acid chromous sulphate solution (M/10 Cr^{++}; pH = 2). The reduced state of the solution was maintained by the presence of amalgamated zinc prepared according to the methods of Stone and Beeson (1936). Gas exchange with the solution was facilitated by use of a sintered glass bubbler which broke up the gas stream into very small bubbles. After the air was washed out of the excystment chamber with this nitrogen and sufficient time elapsed so that equilibrium between the gaseous and liquid phase was approached, then excystment solution which had been de-oxygenated was added. There were no signs of excystment for periods of as long as 25 hours, though at 15 mm. Hg partial oxygen pressure, excystment at 20° C. would have been completed in two to three hours.

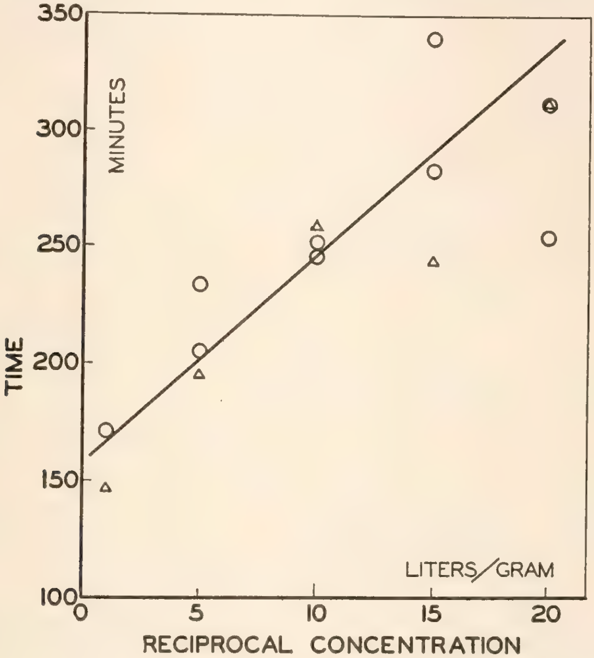

FIG. 2. Excystment of *Colpoda* at 20° C. ○, two control series; △, oxygen tension = 15 mm. Hg.

The data demonstrate much more than just a prevention of excystment by absence of oxygen, for upon admittance of air, normal excystment ensued; and further, the excystment time following the block was found to be independent of the duration of the block, independent of the concentration of the excystment solution, and equal in length to the period that was found from studies on relation between concentration and excystment time to be independent of the concentration of the excystment solution. The experimental data for two of the series of experiments at 20° C. are shown in Figure 3. Detailed tables of these data can be found in Brown (1938*a*).

Apparently the reactions of the period in excystment depending on concentration of the excystment solution go to completion and the reactions of the subsequent periods are completely blocked in the absence of oxygen. These results are in agreement with the hypotheses previously made that the first period is controlled by a diffusion phenomenon and that the following periods at 20° C. are controlled by a reaction of oxidative metabolism (Brown and Taylor, 1938). The fact that the reactions do not proceed in the second period in the absence of oxygen does not in itself prove that the normal limiting reaction is the oxidative metabolism—more refined experiments in which tests are made over a temperature range and in which excystment proceeds, but at a reduced rate due to oxygen tension being a limiting factor, are required.

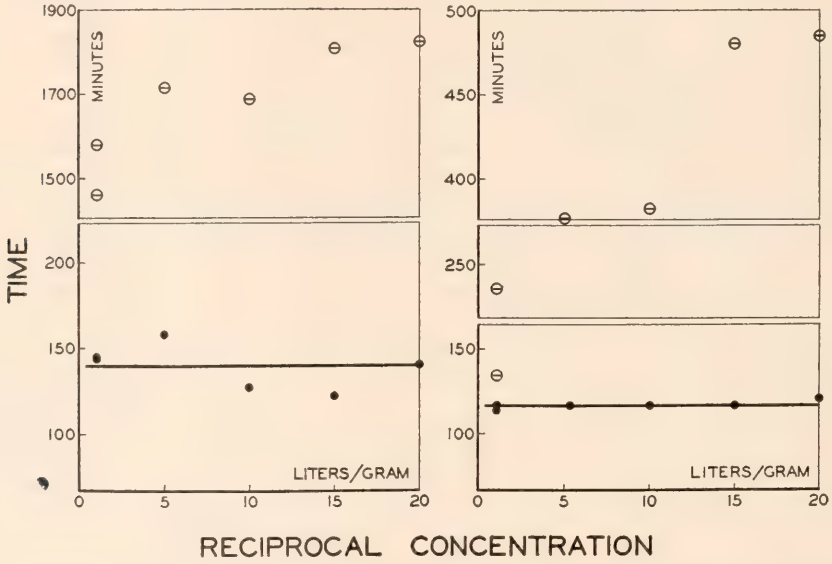

FIG. 3. Excystment of *Colpoda* at 20° C. after being blocked by very low oxygen tension. ●, time between admittance of air and emergence; ⊖, time between addition of excystment solution and emergence.

Though the period dependent on extract concentration changes with temperature in the range 12° to 32° C. as though it were controlled by the time required for diffusion of a substance from the excystment solution, below 12° C. this period changes with temperature according to the Arrhenius equation with a very high μ value (Brown and Taylor, 1938). From this, one might expect that a different process limits this period in the low temperature range. However, this other process, if it exists, is also independent of oxygen, for when the experiments were repeated at 11° C. it was found that the time after admittance of air for completion of excystment is at this temperature also independent of concentration and equal in duration to the period which is found by study of the relation between excystment time and concentration to be independent of concentration. Two of the low temperature series are shown in Figure 4. In the second of these series shown, the set of six dishes included four which were blocked by absence of oxygen and two to which the excystment solution was added at the time of admission of air; the conditions in the experimental and control dishes seem much more comparable in this case for it is seen that the extrapolated value for duration of the period independent of concentration coincides much more closely with the time for excystment after the block than in the cases in which the controls were run separately.

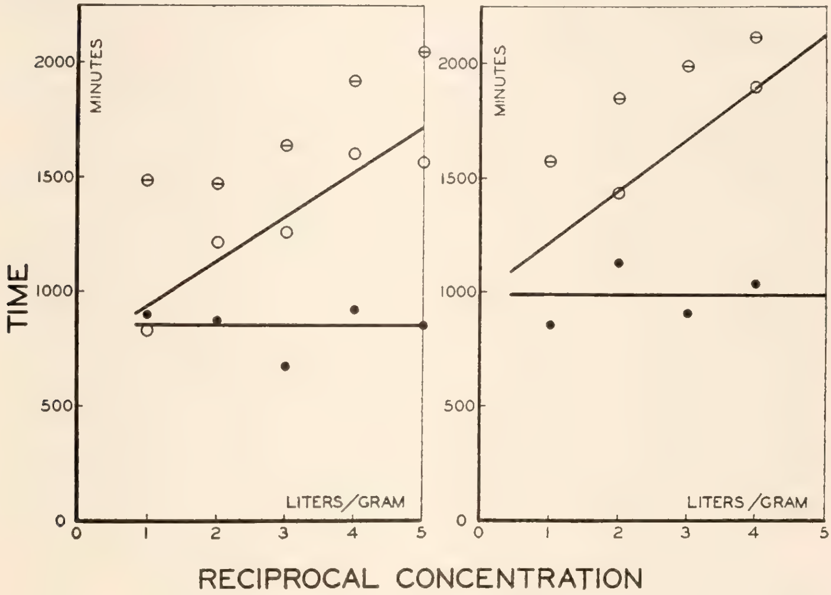

FIG. 4. Excystment of *Colpoda* at 11° C. after being blocked by very low oxygen tension. ●, time between admittance of air and emergence; ⊖, time between addition of excystment solution and emergence; ○, control-time between addition of excystment solution under aerobic conditions and emergence.

DISCUSSION

At present four physiological periods in excystment of *Colpoda* have been sorted out by a quantitative study of excystment time under a variety of environmental conditions. Separation and characterization of the first and subsequent periods is by Brown and Taylor (1938) and this report and separation of the later periods is through the work of Taylor, Brown, and Strickland (1936) on the effects of x-ray irradiation at different stages of excystment.

The experimental characterization and physiological interpretation of these periods is briefly as follows:

I. A period whose duration is inversely proportional to the concentration of the organic constituents of the excystment solution (Brown and Taylor, 1938), and independent of oxygen tension (this report). Its duration changes with temperature as does the viscosity of the cytoplasm for a considerable temperature range (Brown and Taylor, 1938). This period is considered to be one during which diffusion of essential substances from the excystment solution takes place and possibly also an anaerobic reaction with high activation energy (Brown and Taylor, 1938, and this report).[3]

II + III + IV. A period whose duration is independent of the concentration of the excystment solution, and which is dependent upon oxygen (Brown and Taylor, 1938, and this report). The change in duration with change in temperature follows the Arrhenius equation with $\mu = 44{,}000$ calories/ mole below 15° C., 18,000 calories/mole between 15° and 25° C., and zero above 25° C. (Brown and Taylor, 1938). It is suggested that the value of $\mu = 18{,}000$ is associated with oxidative metabolism and $\mu = 44{,}000$ with an anabolic reaction of excystment (Brown and Taylor, 1938).

II. A period during which x-ray irradiation increases excystment time to the same extent as does irradiation at any time in Period I (Taylor, Brown, and Strickland, 1936).

III. A short period during which emergence from the cyst is prevented by the same x-ray dose that at other periods only delays excystment or has no effect (Taylor, Brown, and Strickland, 1936). This period seems to be critical to the later building up of hydrostatic pressure which results in rupturing of the ectocyst membrane, for the irradiation prevents emergence but does not prevent the completion of differentiation of cilia or their functioning (unpublished observations of Taylor, Brown, and Strickland; see also Brown, 1938*a*).

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[3] A recent abstract of a paper by Danielli (1939) not yet published indicates that one might expect the rate of diffusion through living cell membranes to change with temperature according to the Arrhenius equation. This suggests that below 12° C. diffusion is the limiting factor in this first period in encystment rather than some postulated chemical reaction (Brown and Taylor, 1938) but that the mechanism limiting diffusion above 12° C. and below is different, i.e., a barrier of the type suggested by Danielli which requires diffusing molecules to possess greater than a certain kinetic energy in order to penetrate into the cell is limiting below 12° C., whereas cell structures that control diffusion rate according to their viscosity are limiting above 12° C.

IV. A period throughout which administration of an x-ray dose which caused a three-fold increase in excystment time if given during Periods I or II and prevented emergence if given in Period III has almost no influence on excystment time (Taylor, Brown, and Strickland, 1936). This period is considered separated from preceding ones by completion of a developmental reaction involving a substance which may be inactivated by x-ray irradiation during any preceding period.

That *Colpoda* blocked from excystment by absence of oxygen do not die or show any adverse effects for a block of at least 25 hours at 20° C. is opposite to the interpretation of some experiments with free-swimming *Colpoda* (Taylor and Strickland, 1938). In these experiments it was observed that free-swimming organisms die within a short time (97 per cent in two hours) in an unaerated dense bacterial suspension but do not die in a similar suspension which is aerated. From this it was concluded that low oxygen tensions cause death of free-swimming *Colpoda* within a few hours. This may indicate that certain enzyme systems which may be thrown out of balance by removal of oxygen and which then destroy the free-swimming organism are not activated until the second or later periods of excystment.

SUMMARY

1. Excystment time is independent of oxygen tension down to 15 mm. Hg.

2. Excystment is blocked by very low oxygen tensions. This block is at a developmental stage between the excystment period dependent on concentration of the excystment solution and the periods independent of concentration.

3. The excystment process may be divided into four physiological periods characterized by the influence of temperature, concentration of the excystment solution, oxygen tension, and x-ray irradiation on the excystment time.

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THE RELATION BETWEEN KIND OF FOOD, GROWTH, AND STRUCTURE IN AMOEBA[1]

S. O. MAST

(*From the Zoölogical Laboratory of the Johns Hopkins University and the Marine Biological Laboratory, Woods Hole, Mass.*)

It is well known that amoebae usually feed on living organisms and that they ordinarily ingest several different kinds. It has, however, been demonstrated that for some species, one kind suffices for growth (Oehler, 1916, 1924; Rice, 1935; Hopkins, 1937). No observations have been made on the relation between the kind of food and the structure of amoebae. This is the main problem involved in the following experiments.

Amoeba proteus and *Amoeba dubia* grown in Hahnert solution[2] containing rice grains were fed on *Chilomonas paramecium* raised on sterile acetate-ammonium[3] and glucose-peptone[4] solutions respectively and *Colpidium striatum* raised on sterile tryptone-phosphate[5] solution. The experiments were made as follows:

Numerous amoebae were taken from vigorous cultures, passed through several separate portions of distilled water so as to remove the food, and then left in distilled water several hours. In this, many of them became stellate in form. The largest of these were selected and five of them put into each of four 6 cc. glass salt dishes containing 3 cc. Hahnert solution each. Then numerous chilomonads or colpidia which by means of the centrifuge had been passed successively through 4 separate portions of fresh Hahnert solutions were added to the solution in each, and left two hours, i.e. until the amoebae had ingested many chilomonads or colpidia, then the amoebae with as little solution as possible were transferred to clean salt dishes containing Hahnert solution. This was repeated until the solution was free of chilomonads or colpidia, after which the process of feeding and transferring was repeated and the number of amoebae in each dish recorded daily for 9 days, then 5 of the specimens in each dish were transferred to clean dishes containing Hahnert solution and the rest discarded or used for the study of structure, after which the process of feeding, transferring, and recording was again repeated daily for 16 days. The results obtained are presented in Table I.

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[1] I am much indebted to Drs. R. A. Fennell and William J. Bowen for very efficient assistance in the experimental part of this work.

[2] Hahnert solution—KCl, 4 mg.; $CaCl_2$, 4 mg.; $CaH_4(PO_4)_2$, 2 mg.; $Mg_3(PO_4)_2$, 2 mg.; $Ca_3(PO_4)_2$, 2 mg.; water, 1000 cc.

[3] Acetate-ammonium solution—$NaC_2H_3O_2$, 150 mg.; NH_4Cl, 46 mg.; $(NH_4)_2SO_4$, 10 mg.; K_2HPO_4, 20 mg.; $MgCl_2$, 1 mg.; $CaCl_2$, 1.16 mg.; water, 100 cc.

[4] Glucose-peptone solution—peptone, 8 g.; glucose, 2 g.; water, 1000 cc.

[5] Tryptone-phosphate solution—tryptone, 15 g.; KH_2PO_4, 2 g.; water, 1000 cc. 1000 cc.

TABLE I

Growth of Amoeba fed on chilomonads and colpidia respectively. Temperature, 21°–25° C.; *x*, all but five discarded; * Several specimens removed for study of structure.

Food	*Amoeba proteus*											
	Number of Specimens											
	August										September	
Chilomonas in glucose-peptone solution	7	10	12	14	16	16	20	23	26	29	1	4
	5	8	8	9	6^x	5	6	13	12	11	12*	
	5	9	12	15	11^x	5	6	7	7	10	9*	
	5	7	6	7	6^x	5	5	7	7	10	9*	
	5	6	7	4	3^x	3	0					
Chilomonas in acetate-ammonium solution								5	3	0		
								5	7	0		
								5	7	1		
Colpidium	5	16	24	34	35^x	5	12*	17	26	29	42*	7
	3	6	16	23	16^x	5	8	13	14	20	23*	24
	4	12	24	50	90^x	5	8	9	5	11	15	20
	5	8	17	27	45^x	5	7	7	11	18	29	40
	Amoeba dubia											
Chilomonas in glucose-peptone solution	5	6	4	2				5	5	6		
	5	4	3	5				5	5	0		
	5	7	5	4				5	6	3		
	5	4	5	5				5	5	0		
Chilomonas in acetate-ammonium solution								5	2	0		
								5	3	0		
								5	5	2		
Colpidium	5	10	13	17	22^x	5	5	5	2	0		
	5	13	24	26	44^x	5	3	6	2	1		
	5	9	11	13	21^x	5	6	3	3	0		
	5	12	13	12	20^x	5	1	0				

This table shows that both *Amoeba proteus* and *Amoeba dubia* fed exclusively either on chilomonads or colpidia increased in number, but that the increase continued thruout the experiment only in *Amoeba proteus* fed on colpidia. It shows that some of the specimens of *Amoeba proteus* fed on chilomonads were still alive at the close of the experiment, but that the number had decreased; and microscopic examination showed that they were in very poor condition. The table shows that the specimens of *Amoeba dubia* fed on colpidia increased in number much more rapidly and lived much longer than those fed on chilomonads and it shows that for several days those fed on colpidia increased in number as rapidly as *Amoeba proteus* fed on these organisms, but that they then decreased rapidly in number and soon died. The table shows that no increase in number occurred in the specimens of either of the two species of *Amoeba* fed on chilomonads grown in acetate-ammonium solution and that they did not live so long as those fed on chilomonads grown in glucose-peptone solution.

This experiment was repeated in part several times. In some of the tests made, the colpidia used were taken from a culture which contained an unidentified mold, but no bacteria. In some of these tests, the amoebae were left with the food 2 hours, i.e. the same length of time as in the preceding experiments, but in others they were left only 15 minutes and in still others they were left 24 hours.

In the tests in which the amoebae were left with the food only 15 minutes there was no increase in number, either in those fed on chilomonads or in those fed on colpidia. The time was obviously not long enough for the amoebae to ingest sufficient food for growth. The results obtained in the tests in which the amoebae were left with the food 2 and 24 hours respectively are essentially the same as those presented in Table I. That is, in the tests in which chilomonads were used as food, the amoebae usually increased in number fairly rapidly for several days and then decreased, and in those in which colpidia were used, the increase in number continued longer and, under some conditions, doubtless would have continued indefinitely if the tests had not been closed. For example, in one test with *Amoeba proteus* fed on chilomonads, the number increased from 5 to 330 in ten days after which there was a slight increase for a few days, then a gradual decrease to zero, and in another with *Amoeba proteus* fed on colpidia there was a slow, but consistent increase in number for 34 days, i.e. thruout the entire experiment, with no indication of deterioration whatever, altho the increase during this entire time was only from 10 to 106.

The results obtained seem to demonstrate therefore that *Amoeba proteus* can grow and live indefinitely on sterile colpidia as food, but that *Amoeba dubia* cannot, and that neither can live indefinitely on chilomonads as food, but that chilomonads grown in glucose-peptone solution are more nearly adequate as food than those grown in acetate-ammonium solution.

The chilomonads grown in acetate-ammonium solution contained much starch and little fat, while those grown in glucose-peptone solution contained considerable starch but no fat and they were much smaller than the former (Fig. 1). The difference in their food value is, therefore, doubtless due to difference in their chemical structure and content correlated with the chemical composition of the medium in which they grow. Growth in *Amoeba* is consequently not only correlated with the kind and the quantity of organism they ingest, but also with the physiological condition of the organism ingested.

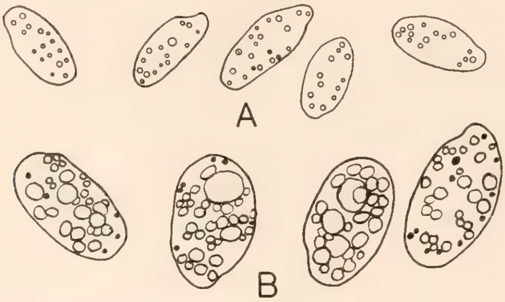

FIG. 1. Camera outlines of *Chilomonas paramecium* showing the effect of the kind of food in the culture medium on size and content. *A*, specimens taken at random from a vigorous culture in sterile glucose-peptone solution; *B*, specimens taken at random from a vigorous culture in acetate-ammonium solution; ○, starch; ●, fat. The flagella are not represented.

Note that the chilomonads grown in glucose-peptone solution were much smaller and contained much less starch and fat than those grown in acetate-ammonium solution. Growth is more rapid in the former solution than in the latter.

In the experiments on growth in *Amoeba* fed exclusively on sterile chilomonads and colpidia respectively, specimens were taken from the cultures at different times and studied in reference to behavior and structure. The results obtained are summarized in the following pages.

The specimens of *Amoeba proteus* which had fed exclusively on colpidia for several days were extraordinarily large (Fig. 2) and literally packed full of globules of fat, especially those which had fed on colpidia from the culture which contained mold.[6] They had only a few pseudopods and these were very short, thick, and blunt without a hyaline cap. They were only slightly attached to the substratum and moved about very slowly and irregularly, now in one direction, then in another, giving the impression of very sluggish, aimless, rolling about. Many had two nuclei. The alpha and beta granules were normal in number and structure, but the bipyramidal crystals were scarce and much shorter and thicker and more truncated than usual and there were usually only a few spherical bodies and some specimens had none at all. The spherical bodies were with few exceptions very small and the substance in them undifferentiated. There was nothing in them similar to the fragile shell usually found and all the substances in them usually stained crimson with neutral red, but the central portion often appeared lighter

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[6] This mold contained much fatty acid but no neutral fat and the colpidia contained enormous quantities of neutral fat but no fatty acid. In fresh cultures the colpidia multiplied rapidly and became abundant in 24 hours. At this time the solution was perfectly clear and the colpidia in it contained but little or no fat. Then the solution gradually became turbid and in 4 or 5 days, mold was clearly visible and at this time the colpidia were well filled with globules of fat and each one usually contained 3 or 4 fragments of mold hyphae or spores which contained liberal quantities of fatty acid which was doubtless changed to neutral fat in the cytoplasm of the colpidia.

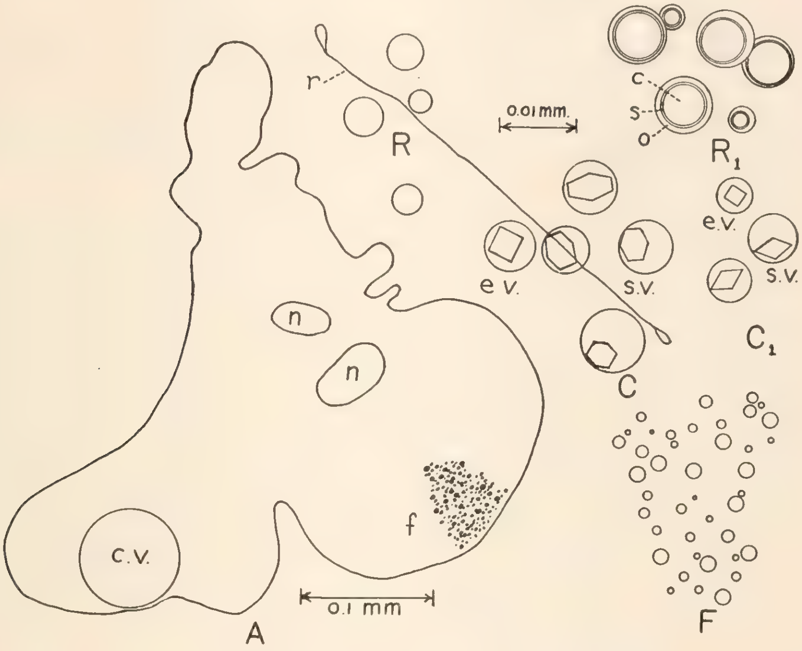

FIG. 2. Camera outlines showing the size, form, and structure of *Amoeba proteus* fed exclusively on colpidia and chilomonads respectively.

A, optical section of *Amoeba proteus* fed on colpidia; *n*, nucleus; *c.v.*, contractile vacuole; *f*, fat globules in one focal plane; *F*, same, enlarged; *C*, bipyramidal crystals in *A*; *R*, largest refractive bodies in *A* (substance in them not differentiated); *r*, one of these drawn out in the form of a fiber; C_1, bipyramidal crystals in a specimen fed on chilomonads; *s.v.*, side view; *e.v.*, end view; R_1, refractive bodies in a small area in an optical plane in a specimen fed on chilomonads (substance in these highly differentiated); *o*, outer layer; *s*, shell; *c*, central substance; *mm*, projected scale.

in color and somewhat more granular than the rest, and did not stain so readily. This substance was usually so elastic that if the bodies were released after they had been flattened by means of pressure on the cover-glass they soon assumed their original shape and it was so adhesive and viscous that if the cover-glass was pushed sidewise on the slide after the bodies had been flattened by pressure on it, the substance in them, owing to adhesions to the glass, was often drawn out in the form of a long slender fiber (Fig. 2).

It is consequently obvious that if the food of specimens of *Amoeba proteus* is restricted to colpidia great changes occur in them in reference to size, form, behavior, and structure; in fact, changes so great that if such specimens were examined without information as to their origin they would certainly be designated as a new species and probably as a new genus.

Specimens of *Amoeba proteus* which for several days had fed exclusively on chilomonads were normal in size, form, and activity; but they contained an extraordinarily large number of spherical bodies (often a thousand or more) and numerous bipyramidal crystals and very little or no fat. The spherical bodies were relatively very large and the substances in them well differentiated into a central mass surrounded with a prominent fragile shell which was covered with a thin layer of oily substance (Fig. 2). In solutions containing neutral red, the outer layer became deep red (crimson) in color, but the central portion and the shell did not stain. The spherical bodies in these amoebae were, therefore, similar to some described by Mast and Doyle (1935, p. 167) but differed radically in number, size, and structure from those found in the amoebae fed exclusively on colpidia.

The bipyramidal crystals were relatively long and but little truncated and in some specimens as many as 2 percent of them were not truncated at all (Fig. 2).

The facts that there were many more refractive bodies in the amoebae which had fed on chilomonads than in those which had fed on colpidia and that they were much larger and the substance in them much more differentiated, show that these structures are closely correlated with the kind of food ingested. They therefore support the contention of Mast and Doyle (1935, p. 291) and others that they are cytoplasmic inclusions and not cytoplasmic structures, i.e. secondary nuclei (Calkins, 1905), cysts (Taylor, 1924), Golgi bodies (Brown, 1930), mitochondria, (Horning, 1925, 1928), vacuome (Volkonsky, 1933).

Amoeba dubia usually contains relatively few crystals (some irregular or roughly bipyramidal in form with the edges and corners rounded, and some thin rectangular plate-like in form) and not much fat.

In the specimens fed on colpidia the irregular crystals decreased greatly in number and often disappeared entirely and the plate-like crystals increased considerably and there was marked accumulation of fat, altho not nearly so much as in *Amoeba proteus.* In those fed on chilomonads the irregular crystals increased greatly in number and the plate-like crystals decreased considerably and the fat usually disappeared.

There was no significant change in size, form, or activity in those fed on colpidia or those fed on chilomonads.

SUMMARY

1. If specimens of *Amoeba proteus* are fed exclusively on colpidia, they become very large and extremely fat and sluggish and grow and multiply slowly, but indefinitely. The refractive bodies in them decrease greatly in number and size and their content becomes homogeneous and very adhesive, elastic and viscous. The crystals decrease in number and become shorter and more truncated.

2. If they are fed exclusively on chilomonads, they grow and multiply for several days, then decrease in number and soon die, but they live longer if the chilomonads have grown in glucose-peptone solution than if they have grown in acetate-ammonium solution. The refractive bodies increase greatly in size and number and the content of these bodies becomes sharply differentiated; the bipyramidal crystals increase in number and become less truncated, and the fat decreases in quantity.

3. If specimens of *Amoeba dubia* feed exclusively on chilomonads, they multiply for a few days, then cease and soon die. The irregular-shaped crystals increase and the plate-like crystals decrease considerably in number and the fat disappears.

4. If they feed exclusively on colpidia, they multiply more and live longer than if they feed exclusively on chilomonads, but they do not live indefinitely. The plate-like crystals increase in number and the irregularly shaped crystals usually disappear entirely and the fat increases in quantity, but not so much as it does in *Amoeba proteus.*

5. *Amoeba* is in reference to form, size, behavior, and structure closely correlated with the kind of organisms it eats and their physiological condition.

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THE EFFECT OF ELECTRIC CURRENT ON THE RELATIVE VISCOSITY OF SEA-URCHIN EGG PROTOPLASM[1]

C. A. ANGERER[2]

(From the Zoölogical Laboratory, University of Pennsylvania,[3] *and the Marine Biological Laboratory, Woods Hole, Mass.)*

Becquerel (1837) was the first investigator to study the action of electricity on protoplasm of single cells. [The greater part of the literature treating of the effect of electric current on cyclosis may be obtained from Ewart (1903).] The conclusion to be drawn from the literature is that electric current, depending on the current density employed, produces a progressive decrease in, followed ultimately by cessation of, cyclosis providing the current flows for a sufficient interval of time. However, Velten (1876), Ewart (1903) and Koketsu (1923) observed an initial increase prior to the characteristic progressive slowing of cyclosis. The results obtained by Brücke (1862) on human leucocytes, Chifflot and Gautier (1905) on *Cosmarium,* Bayliss (1920) on *Tradescantia* and *Amoeba* by the Brownian movement method and Bersa and Weber (1922) on *Phaseolus* by the centrifuge method are in essential agreement, i.e., electric current produces an increase in protoplasmic viscosity.

This investigation was undertaken to continue and extend the study of electric current as a stimulating agent to some type of protoplasm other than that of the protozoan cells *Amoeba dubia* and *A. proteus* already studied (1937).[4] It is of interest to know whether the protoplasm of such distantly related biological groups, e.g., *Amoeba* and the unfertilized eggs of *Arbacia,* responds to this stimulating agent in a comparable manner.

MATERIAL AND METHODS

The experiments were performed on the unfertilized eggs of the sea-urchin, *Arbacia punctulata.* The eggs were treated according to method "3" as described by Just (1928). Eggs were shed in about

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[2] Now of the Dept. of Physiology, The Ohio State University, Columbus.

[3] I wish to express my profound gratitude to Professor C. E. McClung for his many kindnesses during the present investigations.

[4] Our own studies on amoebae are now nearing completion and a detailed report will appear shortly.

250 cc. of sea water and washed once in an approximately equal volume of the medium.

The eggs were subjected to either direct or alternating electric current, according to the experiment in question, in a celluloid trough, the sides of which were perfectly milled and thus parallel to the lines of current flow. The current was applied through a $Zn/ZnSO_4/$ sea-water-in-agar system, which, in turn, was in circuit with a reversing switch, rheostat and milliammeter. The agar bridges were cut to fill the ends of the trough completely and were finally sealed in place by means of hot agar. The available electrode surface, i.e., the cross-sectional area of the available medium in the trough, was 40 mm^2. The source of the electric current was the regular service line (110 volts) running into the laboratory.

The protoplasmic viscosity was determined by the centrifuge method. The handle of an Emerson hand centrifuge, when turned at the rate of one revolution per two seconds, developed a centrifugal force of 2,531 times gravity, after allowance was made for the depth to which the eggs settled in the centrifuge tube when cushioned on an isosmotic (0.73 m.) sucrose solution. The eggs were centrifuged until 80 per cent, or more, showed a fine hyaline band 2/15 the diameter of the egg appearing between the oil cap and the yolk granules. (This fraction was equal to one division of the arbitrary scale of the ocular micrometer employed in these experiments.) The time in seconds necessary to move the yolk granules the specified distance, and thus show the requisite width of the hyaline band at the centripetal pole of the egg, is designated the "centrifuging value." This is the end-point to which all experimental centrifugalizations are referred.

The experiments were conducted in the following manner. Each batch, i.e., eggs from one female, was tested for 'normalcy.' A batch of eggs was declared 'normal' if, on sampling, 95 per cent or more showed membrane elevation after insemination and 80 per cent or more, showed the desired width of the hyaline area when centrifuged for 60 seconds at room temperature (19°–24° C.). These conditions prevailing, approximately uniform quantities of eggs were placed in the stimulating trough and while the eggs were more or less suspended, an electric current of known intensity and duration of flow was admitted. At the cessation of the current the eggs were immediately pipetted into the centrifuge tube, which contained a known depth of isosmotic sucrose solution, and were centrifuged respectively for various known periods of time. The lowest value to which the interval of time elapsing between the cessation of the application of the stimulating agent and incipient centrifugalization could be reduced was 7 seconds. There was no apparent difference between this and the 10-second interval which was used throughout these experiments unless otherwise stated.

The points of curves *A* and *B* (Fig. 1) which represent centrifuging values plotted as functions of the time of exposure to the electric current in question were obtained in the following manner. After preliminary tests in which various constant current densities were studied, when applied for varying intervals of time, it was apparent that a current density [5] of 0.005 amperes/mm.^{2} served best to illustrate the results and this density was employed throughout these experiments.

On the basis of the above procedure the curves were developed as follows. In the early experiments several batches of eggs were necessary in order to work out a complete curve. With more experience one batch of eggs sufficed for the determination of the points for any one curve. Twelve curves for direct (*B*) and ten curves for alternating (*A*) current were thus worked out. Finally, with these data as a basis, all points for both alternating and direct current curves were obtained by employing one batch of eggs. This procedure was repeated for three different batches of eggs and thus assured relatively constant conditions of material, temperature (21.5° C.), etc. Hence fewer experiments were performed in the latter instance but these data were substantiated by the

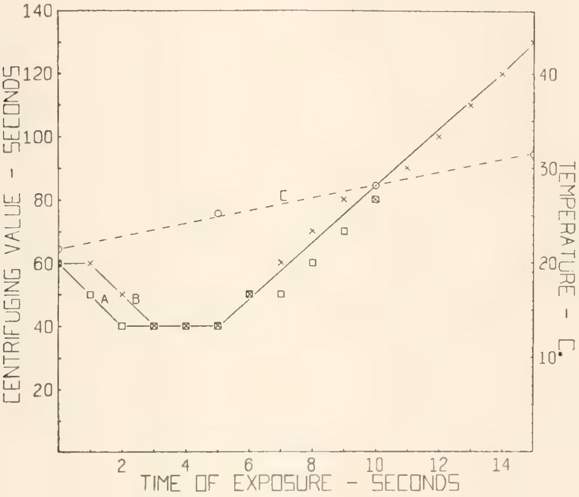

FIG. 1. Centrifuging time in seconds (viscosity) of *Arbacia* egg protoplasm vs. time of exposure in seconds to alternating (curve *A*) and direct (curve *B*) electric current. Curve *C* represents the rate of thermal increase to which the stimulating trough is subjected vs. time of exposure to either type of current. Current density = 0.005 amperes/mm.^{2}; pH 8.2; room temperature = 21.5° C.

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[5] In a preliminary note (1938) amperes/cm.^{2} should have read amperes/mm.^{2}.

more detailed experiments performed during the early part of the work. Under the conditions of the experiments no point on the curves deviated more than ten arbitrary units in centrifuging value in repetitive tests.

Eggs were not used for experimental purposes after being shed longer than three hours. The viscosity of the main protoplasmic mass, as determined by the method here employed, does not undergo any appreciable change during this three-hour period (Goldforb, 1935; Angerer, 1937).

There was no observable difference when experimentally treated eggs were compared with their controls for membrane elevation and cleavage.

RESULTS

Direct Current

When sea-urchin eggs are subjected to a current density of 0.005 amperes/mm.^{2} flowing for varying known intervals of time, there is, in the majority of experiments, no observable change in the centrifuging value (curve B) after one second of continuous exposure.[6] On continuous application of the current for two seconds, there is, in all experiments, a decrease in the centrifuging value; while with three seconds of continuous exposure the viscosity decreases from the control centrifuging value of 60 to a minimum value of 40 arbitrary units, i.e., a decrement of 33 per cent in three seconds. There is no further change in viscosity for the next four or five seconds, respectively, of continuous exposure to the current.[7] However, if the current is allowed to flow for six seconds, the centrifuging value increases from a transient minimum value until after seven seconds of constant exposure the centrifuging value is identical with that of the control. There is a progressive increase on further exposure, so that after fifteen seconds, when these experiments were discontinued, the centrifuging value had increased 225 per cent above the previous minimum value.

Alternating Current

When the centrifuging values are plotted as functions of the time of exposure in seconds (curve A) to alternating current of 0.005 amperes/

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[6] In a few experiments the viscosity at the end of this period of time was found to show a slight decrease which was never greater than a centrifuging value of ten seconds.

[7] Occasionally eggs, after exposure to electric current, showed a tendency for the intracellular granules to stick in the cortical area. This condition, though infrequent, was confined, more particularly, to eggs from certain females. Though these eggs were discarded in the final count, since the behavior of the main mass of the protoplasm was of chief interest, their number was not of such magnitude as to affect appreciably the results.

mm.^{2} there is, in all experiments, after one second of constant current flow a decrease in the viscosity of the protoplasm as measured by the centrifuging value. This value is decreased further when the eggs are exposed to the current for two seconds, while after three seconds the centrifuging value undergoes no further decrease but levels at the new minimum value which is 67 per cent of the control. This minimum value is maintained after continuous exposure for three, four and five seconds, respectively.[7] Continuing the exposure to the stimulating agent further, there is, after five seconds, a perceptible increase in the viscosity value. Thereafter, with each successive second of exposure to the electric current a progressive increase is noted in the centrifuging value. The increment is of the same value as that recorded for direct current (curve *B*), namely 225 per cent in ten seconds. The results recorded (curve *A*) were discontinued in this experiment after ten seconds exposure to alternating current because of the expiration of the time limit set upon the use of shed eggs.

It was of interest, in view of the high resistance at the sea water-agar interface, to determine the thermal change occurring within the stimulating trough as a result of the passage of an electric current of specified density. The main coördinates of the broken-line curve *C* (labeled at the right and lower sides of Fig. 1) represent temperature as a function of time during which the eggs contained in the trough are exposed to the thermal effect induced by passage of the electric current. The temperature data were obtained by a specially constructed, direct reading thermometer, the bulb of which was of such size as to be submerged completely when immersed in the stimulating trough.

That thermal effects of the magnitude present during the course of these experiments have no observable effect on the centrifuging value is shown on immersing eggs in sea water which has been warmed previously to 32° C. When eggs are exposed to this temperature for greater intervals of time (e.g., 20 seconds) than that to which they are subjected during the course of these experiments and are simultaneously centrifuged with eggs serving as controls, it is found that the centrifuging values are identical. Heilbrunn (1924), though not primarily interested in this phase of the question, states in the protocol (p. 192) of his experiments on heat coagulation in sea-urchin eggs that after five minutes exposure to 32.9° C. the heat-treated eggs were found to show the same width of the hyaline area as the controls.

Experiments were conducted to test whether varying the quantity of eggs suspended in unit volume of sea water in the specified electric field had any tendency to alter the shape of the electric current-viscosity curves. Batches of eggs were allowed to settle under the influence of gravity in a four-inch finger bowl and minimum amounts of sea water plus the relatively concentrated eggs were picked up by means of a regular medicine dropper. Various points on the electric current-viscosity curves were investigated using one, ten and twenty drops respectively of the egg suspension in unit volume of bathing medium. In the various concentrations of eggs employed there is no observable difference in the centrifuging values other than that which is within the range of experimental error.

Discussion

When either direct or alternating electric current of the intensity employed in these experiments is used as a stimulating agent, there is initially a transient decrease followed by a progressive increase in the viscosity of the protoplasm of sea-urchin eggs. An ultimate increase in the centrifuging value is in line with the literature (see introduction); though no lucid evidence is to be found in favor of a transitory decrease in viscosity prior to the ultimate increase.

In view of Heilbronn's (1914) results on the attempt to correlate cyclosis in terms of viscosity data, it is justifiable to consider only, at the present time, data as obtained from the methods of Brownian movement and centrifugalization. Brücke (1862), Kühne (1864), Chifflot and Gautier (1905), and Bayliss (1920) have observed a decrease or stoppage of Brownian movement on passage of an electric current through the cell. These data, insofar as a definite statement as to the experimental procedure is given, were obtained during the actual passage of electric current through the material in question and not immediately thereafter as in the experiments here reported. There may be some criticism of studying Brownian movement during the actual passage of the current since cytoplasmic granules undergoing electrophoresis lose their characteristic trembling movements (unpublished results). Mast (1931) should be consulted in this connection. Bersa and Weber (1922), using the centrifuge method, observed an increase in the viscosity of the protoplasm of *Phaseolus* on the passage of electric current for relatively long periods of time. It would be of interest to ascertain data for shorter intervals of time.

There is no difference in the results whether one employs alternating or direct current providing identical current densities are employed (compare curves *A* and *B*). Alternating current tends to be more effective initially owing, apparently, to the greater shearing effect produced by the protoplasmic granules suspended in an oscillating electric field which would tend to break down the protoplasmic structure. This effect may be reenforced by the apparently thixotropic character of protoplasm. For a review of the literature on thixotropy in living cells see Angerer (1936).

The question arises as to the congruity of applying a stimulating agent for the duration of a few seconds while a minimum of 50 seconds is required for obtaining the viscosity determination. When varying intervals of time are permitted to elapse from the cessation of the electric current to incipient centrifugalization, the results obtained are found not to vary for at least two minutes.

The data presented here are in accord with the known facts concerning the action of certain stimulating agents on sea-urchin egg protoplasm. Heilbrunn and Young (1930) and Angerer (1937),[8] employing respectively ultra-violet radiations and mechanical agitation, found a transitory liquefaction prior to an ultimate increase in viscosity. Similar results were obtained for ultra-violet radiations (Heilbrunn and Daugherty, 1933), mechanical agitation, electric current and suddenly applied thermal increments (Angerer, 1936, 1938, 1940) on *Amoeba* protoplasm. To explain their results, Heilbrunn and Daugherty (1933) proposed a theory in terms of colloid chemical changes in protoplasm; for a detailed review of this theory one is referred to Chapter 37 of Heilbrunn's book (1937).

SUMMARY

1. The centrifuge method was used to determine the viscosity of sea-urchin egg protoplasm after exposure to either direct or alternating electric current to a current density of 0.005 amperes/mm.^{2} for various known intervals of time.

2. There is, on exposing eggs to either direct (curve *B*) or alternating (curve *A*) current, a transient decrease followed ultimately by a progressive increase in the centrifuging value (Fig. 1).

3. Since the data for the action of electric current, as employed in these experiments, show a striking similarity to those results as obtained by the use of certain other stimulating agents on *Amoeba* and *Arbacia* egg protoplasm, it is suggested that the mechanism offered by Heilbrunn (1937) may be applicable here.

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[8] Footnote p. 340.

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DEVELOPMENT OF EYE COLORS IN DROSOPHILA: PRODUCTION OF v^+ HORMONE BY FAT BODIES[1]

G. W. BEADLE, E. L. TATUM AND C. W. CLANCY

(From the School of Biological Sciences, Stanford University, California)

Of the two diffusible substances known to be concerned in the production of eye pigments in *Drosophila,* only v^+ hormone is produced by fat bodies (Beadle, 1937). This was demonstrated by transplantation experiments. Attempts to extract this hormone from larval fat bodies were unsuccessful and it was therefore concluded that the hormone is produced after the time of puparium formation. It is the purpose of this paper to summarize additional experiments designed to determine when and under what conditions fat bodies produce this hormone. Unless otherwise indicated, fat bodies were taken from wild-type larvae or prepupae. All tests for v^+ hormone were made by using vermilion brown flies as described by Tatum and Beadle (1938). The few tests made for cn^+ hormone were made in a similar way using cinnabar brown flies.

LARVAL FAT BODIES

Although v^+ hormone could not be extracted from larval fat bodies with Ringer's solution at 100° C., it was felt that the hormone as such might nevertheless be present but in such a state that it was not extracted by the method used. Accordingly, several additional methods of extraction have been used.

Fifty sets of dissected fat bodies were heated in distilled water, oven-dried, and extracted with chloroform. The chloroform-insoluble material was taken up in hot Ringer's solution and injected into vermilion brown test larvae. The results were negative (8 flies). Since the hormone is known to be chloroform-insoluble and water-soluble, these tests confirm those previously made. Other tests in which the dissected fat bodies were ground with powdered silica were likewise negative (3 flies). Alternate freezing of fat bodies (in an acetone and solid CO_2 mixture) and thawing for six successive times failed to yield any hormone in subsequent extracts made with hot Ringer's solution (6 flies).

Digestion of larval fat bodies with trypsin failed to liberate any

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[1] Work supported by funds granted by the Rockefeller Foundation.

hormone. In one experiment 20 sets of fat bodies from mature larvae were incubated for 24 hours at 37° C. in 0.03 cc. of a solution of 0.5 per cent of $NaHCO_2$ and 0.025 per cent of trypsin made up in Ringer's. The clear solution obtained after heating and centrifuging through a microfilter gave negative results (20 flies). Appropriate controls showed that under these conditions the trypsin used was active in digesting casein and that it did not alter the activity of concentrated extracts of the hormone. Observations showed that the trypsin-treated fat bodies were visibly broken down.

Further attempts to determine whether any v^+ hormone is present in larval fat bodies were made by freezing such tissues with solid CO_2 and then transplanting them to test animals. This was done by taking up the fat body tissue in a regular transplantation pipette (Ephrussi and Beadle, 1936) and then placing the shaft of the pipette in contact with a small piece of solid CO_2. The temperature actually attained by the tissue itself was not determined; it was without question well above that of the CO_2. Ten flies to each of which a section of fat body (attached along one margin to the salivary gland) had been transplanted after being frozen three times, showed little or no eye-color modification. Other experiments using a more or less similar technique were made with fat bodies immersed in boiling water before transplantation. Considerable difficulty was encountered in doing this, but by coating the inside of the pipettes with a thin film of agar, drawing up the fat-body tissue, and then immersing the pipette in boiling water, a number of successful transplants were made. Of seven test animals to which such heated fat bodies were transplanted, six were quite negative. The seventh showed a color modification of 2.5 (medium response—see Tatum and Beadle, 1938, for significance of color values). This exceptional animal was undoubtedly one to which by mistake an unheated fat body had been transplanted. Because of the technical difficulty of making such transplants such an error could easily have been made. Living fat bodies were transplanted as controls for both the frozen and heated series. Eight such control transplants gave a mean color value of 2.4 (1.8 to 3.0). A single control transplant gave a negative test, presumably due to failure of the operation.

These experiments agree with those previously made and indicate that little or no v^+ hormone is present in larval fat bodies prior to puparium formation, and consequently that the major portion of the hormone produced by such tissues is elaborated after puparium formation. It is possible that a small amount of hormone is produced before this time but in too small an amount to be detected by the methods used.

Fat Bodies of Prepupae

Preliminary experiments indicated that v^+ hormone is present in prepupal fat bodies and can be extracted from them during this stage. Several series of extractions of prepupal fat bodies taken from animals of various ages were therefore made. In each case 20 sets of fat bodies were heated in 0.03 cc. of Ringer's solution and the solution removed by centrifuging through a microfilter. One series, using prepupae from the Oregon-r wild-type stock gave the results shown in Table I.

Table I

Age in Hours after Puparium Formation	Number of Test Animals	Eye Color, Mean and Range
0–1	11	0.1 (0.0–0.4)
3–4.5	14	0.0
6–8	10	2.2 (1.5–3.0)
7–9	15	2.3 (0.0–3.2)
10–11.8	10	0.8 (0.0–2.7)

There is no apparent reason why the 3–4.5 hour prepupal fat bodies gave negative results. A number of other tests indicate that the results are generally erratic for young prepupae. Thus a separate set gave a mean color value of 1.3 for an extract of 0–1 hour prepupal fat bodies. A series of tests of prepupae from the Canton-S wild-type stock gave the results shown in Table II.

Table II

Age in hours	Number	Eye Color
larval	10	0.0
0–1	10	0.3 (0.1–0.6)
1.8–3.5	8	0.7 (0.0–1.6)
8–9.5	8	1.4 (0.7–2.0)

An additional experiment using 10–12-hour Oregon-r prepupae gave a test of 2.3 (6 animals 1.3–3.0). It should be pointed out that the tests of older prepupae are unreliable because of the impossibility of being sure of getting all of the fat body tissue. At this time the fat bodies are undergoing the breakdown process characteristic of metamorphosis.

These results suffice to show that the hormone is present in fat body tissue and may be extracted over most of the prepupal period. Because of the several difficulties involved in such tests as these, the results are only roughly indicative of quantitative relations.

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Correlation of Hormone Production and Puparium Formation

Various attempts have been made to alter the conditions so that larval fat bodies would produce v^+ hormone. Unheated wild-type larval fat bodies were allowed to stand in Ringer's solution for 5 to 6 hours at room temperature. Extracts of these gave negative results. Several series of 48-hour-old wild-type larvae were subjected to semi-starvation conditions by transferring them to 0.25 per cent dry brewers' yeast in 1 per cent agar as described by Beadle, Tatum and Clancy (1938). This reduced food supply prolongs larval life. Extracts of the fat bodies of such delayed larvae made just prior to puparium formation failed to show the presence of hormone. On the assumption that enzymes might be involved in the production of v^+ hormone by the fat bodies, pupal fluid from vermilion brown animals selected from 0 to 30 hours after puparium formation was injected into wild-type larvae 117–124 hours after egg-laying. Three to 7 hours after these injections were made the fat bodies of the hosts were removed and extracted with hot Ringer's. These extracts were negative in tests for v^+ hormone. A similar experiment in which vermilion brown pupal fluid was injected into 92–97-hour wild-type larvae gave negative results in tests of fat body extracts made 23–25 hours later.

A marked delay in puparium formation brought about by subjecting mature larvae to low temperature apparently does not break down the synchronism between hormone production and puparium formation. An experiment in which wild-type mature larvae were kept at 8–10° C. for 18.5 hours showed that a Ringer extract of fat bodies of 0–1-hour-old prepupae taken at the end of this time gave a mean color value of 0.3 when tested in 11 vermilion brown animals. A comparable extract made from prepupal fat bodies from mature larvae kept continuously at 25° C. gave an average color value of 0.6 (11 flies). Considering the low values obtained from these two extracts and the variation (0.1–0.7 and 0.1 to 0.8 respectively), this difference cannot be regarded as significant.

Prepupal fat bodies 0–1 hours after puparium formation apparently do not continue hormone production when explanted to Ringer's solution. In one experiment 20 sets of such fat bodies were placed in 0.03 cc. of Ringer's solution and allowed to stand at 22° C. for 27–28 hours. At the end of this time a hot-Ringer extract gave a color value of 0.4 (range 0.0–0.7, 14 animals). A control series extracted in a similar way immediately on dissection gave a color value of 0.6 (range 0.1–0.8, 11 animals). The explanted fat bodies did not undergo the breakdown processes characteristic of metamorphosis.

Superfemales of *Drosophila* (individuals with 3 X chromosomes and 2 sets of autosomes) are known to show a delay of one to three days in puparium formation as compared with their normal sisters (Brehme, 1937). During this period, subsequent to puparium formation by their sibs, there is relatively little growth of the superfemale larvae. Extracts of fat bodies of such superfemale larvae taken shortly before puparium formation show that v^+ hormone is present at this time. Thus an extract of 20 sets of fat bodies from mature superfemale larvae in 0.03 cc. of Ringer's solution gave a mean eye-color modification of 0.7 (range 0.0–1.0, 10 animals). Extracts of prepupal fat bodies of superfemales are likewise positive. It is clear, then, that under the particular set of developmental conditions of superfemale larvae the synchronization of fat-body hormone production with puparium formation characteristic of normal larvae is broken down. This shows that the two processes are not inseparably associated at least as regards their time sequence. The mechanism by which the two processes are normally related, however, is entirely a matter of conjecture at the present time.

While under none of the environmental and experimental conditions to which normal larvae were subjected was there any appreciable production of v^+ hormone by the fat-body cells prior to puparium formation, the fact that the sequence of these two processes is modified by the genic imbalance characteristic of superfemales suggests that it might be possible to induce the formation of hormone by cells of this tissue before puparium formation in normal larvae if the proper conditions were brought about. Certainly this possibility is not excluded by any of the work reported in this paper.

In order to determine whether or not fat bodies might have any effect on the eye-color hormones in vitro, an experiment was made in which fat bodies were explanted to a Ringer's solution containing partially purified v^+ and cn^+ hormones. As a control, fat bodies heated for several seconds at 100° C. were allowed to stand in a similar solution of the hormones. In both cases the fat bodies were kept in the solution for 4 hours at room temperature. The results are shown in Table III.

Living fat bodies appear to have no significant effect on the hormones in solution. Since the hormones may be inactivated through oxidation in the presence of certain enzymes present in the organism (Thimann and Beadle, 1937), it may be concluded that the fat body either does not contain or does not liberate such enzymes under the conditions of this experiment.

Relation of the Fat Body to the Starvation Effect

It has been shown that low food level at a certain period of development modifies vermilion flies in some manner such that they produce v^+ hormone (Khouvine, Ephrussi and Chevais, 1938; Beadle, Tatum and Clancy, 1938). Normally such flies produce little or no v^+ eye-color hormone. Since this modification evidently must be due to some alteration in metabolism, attempts have been made to determine what tissues or organs might be involved. It has been found that the fat body is modified by subjection of larvae to low food.

Larvae were transferred from full food to low food at about 48 hours after egg-laying and allowed to complete larval development under these conditions. The methods of inducing an eye color modification in this way are described in the papers referred to above. Fat bodies taken from mature larvae which had been subjected to such semi-starvation conditions were transplanted to vermilion brown larval hosts grown under standard full-food conditions. In one experiment in which fat bodies from vermilion brown larvae grown on low food were transplanted, 21 host animals eclosed. Of these, 16 showed an eye-color modification (mean 0.8, range 0.1–2.0). The remaining 5 were negative, possibly because of unsuccessful operations. Since the fat body normally breaks down during metamorphosis there is no easy way of checking for the presence of implanted tissue. In another series fat bodies from vermilion larvae subjected to a low food level were transplanted to vermilion brown test larvae. Ten animals developed and all showed a positive effect of the implant (mean eye color 1.3, range 0.8–1.9).

Table III

	Test for v^+ hormone		Test for cn^+ hormone	
	Unheated	Heated	Unheated	Heated
Number of tests	11	11	8	9
Mean eye color	3.0	3.1	2.5	2.4
Range	2–3.5	3.0–3.5	2.5	2.0–2.5

Since it is well established that fat bodies of fully fed vermilion (or vermilion brown) larvae give negative results when transplanted to vermilion brown hosts, it is evident that low food of the kind used so modifies the fat body that it subsequently produces v^+ hormone. These results have been checked by direct extraction of the hormone from prepupal fat bodies. Extraction of fat bodies of mature vermilion brown larvae that had been subjected to low food conditions yielded solutions that were negative in tests for v^+ hormone. Fat bodies from prepupae (4.5–6.5 hours after puparium formation) were extracted with hot Ringer's solution. This extract gave a slight but definitely positive modification of the eyes of vermilion brown test animals (11 flies, eye color 0.1–0.2). It appears that in such larvae, as in normal wild-type larvae, the fat body produces v^+ hormone subsequent to the time of puparium formation.

Preliminary studies have indicated that subjection of larvae to low food conditions brings about changes in the cytoplasmic inclusions of the fat body cells. These changes may possibly be correlated with the production of hormone by vermilion larvae which have been grown under semi-starvation conditions. Since these investigations are as yet incomplete, discussion of them will be deferred.

Malpighian tubes of wild-type larvae are known to contain v^+ hormone and there is evidence that they produce this substance. In order to determine whether the low food level might also have an effect on these organs, Malpighian tubes from semi-starved vermilion brown (or vermilion) larvae were transplanted to normal vermilion brown test larvae. It was discovered that tubes from larvae subjected to a low food level tend to kill the hosts to which they are transplanted. Presumably the tubes accumulate toxic substances under such conditions. In a preliminary series four mature recipients showed no eye color modification. In this series, however, no dissections were made to determine whether the implant was present. A second series in which sets of four Malpighian tubes from mature semi-starved vermilion were transplanted to vermilion brown test larvae, nine adult recipients were obtained which dissections showed to contain implanted tubes. Eight of these showed a relatively weak eye color response (0.5) indicating that hormone was present or was produced—the ninth was negative.

It appears that the Malpighian tubes of vermilion larvae contain or produce some v^+ hormone under the semi-starvation conditions to which these larvae were subjected. The effect, however, seems to be less strong than that on the fat bodies. It is possible that the hormone released from larval Malpighian tube transplants represents accumulation and is not produced by the tubes themselves. It does not seem probable that the hormone is produced by the fat body, although we have not entirely excluded the possibility that the larval fat body produces hormone at a low rate. The fact that no hormone (or very little) accumulates in the larval fat body argues that if it is produced in this tissue during larval life, it must diffuse out approximately as fast as it is formed.

SUMMARY

Under normal genetic and environmental conditions fat-body cells produce v^+ hormone after the time of puparium formation but not before. Attempts to induce hormone production by fat-body tissue before puparium formation were unsuccessful. Since it is shown that larval fat bodies of mature superfemale larvae contain v^+ hormone, however, it is clear that the normal sequence of puparium formation and hormone production is not a necessary and invariable one.

Active solutions of v^+ hormone are readily obtained by extracting prepupal fat bodies over practically the entire period of prepupal development.

It is shown that the so-called " starvation effect " on eye pigmentation involves a modification of genetically vermilion fat body cells such that they produce v^+ hormone, whereas normally they are unable to do so. It is possible but not definitely established that a somewhat similar modification is brought about in cells of the Malpighian tubes by semistarvation of larvae.

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EFFECT OF DIET ON EYE-COLOR DEVELOPMENT IN DROSOPHILA MELANOGASTER[1]

E. L. TATUM AND G. W. BEADLE

(From the School of Biological Sciences, Stanford University)

Vermilion brown (*v bw*) larvae of *D. melanogaster* placed on a low food level diet produce v^+ eye-color hormone, and therefore, as flies, develop pigmented eyes (Khouvine, Ephrussi and Chevais, 1938). Beadle, Tatum and Clancy (1938) showed that larvae are affected in this way by low food level during a certain sensitive period lying between 60 and 70 hours from egg laying. Khouvine, Ephrussi and Chevais reported that sugar added to the starvation diet inhibits the starvation effect. Their work, however, did not eliminate the possibility that the sugar effect was associated only indirectly with hormone production in the flies, possibly through the intermediation of growing yeast or other micro-organisms. We have investigated the effects under aseptic conditions of various supplements to a low yeast diet on the growth and eye-color development of vermilion brown animals. Under these conditions carbohydrates and related substances inhibit the starvation effect, while proteins and amino acids do not. The present paper summarizes these results.

EXPERIMENTAL

Culture and Methods

The aseptic cultures of vermilion brown larvae used throughout this work were obtained by a slight modification of Baumberger's (1919) alcohol sterilization method. Eggs were collected over a 2- to 3-hour period on freshly autoclaved standard corn-meal molasses agar, without added yeast. Shortly after collection 20 to 30 eggs were picked up on a single small sterilized glass rod flattened at the end. The rods with the eggs were then placed individually in small sterile vials containing 85 per cent alcohol. After 10 minutes the rod was removed and the eggs were pushed off onto the sterile test medium, using ordinary bacteriological methods to insure sterility. All cultures were incubated at 25° C. unless otherwise stated.

The standard starvation food contained 1.5 per cent agar and 0.5 per cent Fleischmann's dry brewers' yeast made up with distilled water.

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Ten cc. of this mixture and the desired amounts of the various supplements were placed in 35 cc. vials which were stoppered with cloth-covered cotton plugs. After sterilization in the autoclave the vials were cooled and agitated, and finally slanted so that the solid yeast remained suspended throughout the medium. Routine checks of sterility were made after pupation of the larvae by streaking a loopful of the medium onto yeast extract-glucose agar. Any vials which were not bacteriologically sterile at this time were discarded.

Cultures were observed every 24 hours, so that the time to emergence of the flies was accurate only within this period. The observed prolongation of larval and pupal life as compared with the normal 215 hours on full food is given in days from egg-laying. The delay actually represents prolongation of larval life, since Beadle et al. (1938) showed that duration of pupal life is practically constant under all conditions. After emergence of the flies the intensity of pigmentation of the eyes was graded according to the scale of eye-color values described by Tatum and Beadle (1938). These values have a definite relation to the amount of hormone available to the fly, but for simplification all results are given only as color values. It should be remembered that the increased intensity of eye pigmentation resulting from starvation involves the actual production of v^+ hormone (Beadle et al., 1938).

TABLE I

Influence of yeast concentration and temperature on the starvation effect. Basic medium: 1.5 per cent agar.

Temperature °C.	Yeast concentration (per cent)											
	0.5			1.0			3.0			5.0		
	Days to emergence	No. of adult flies	Eye color	Days to emergence	No. of adult flies	Eye color	Days to emergence	No. of adult flies	Eye color	Days to emergence	No. of adult flies	Eye color
17°	22–25*	7	3.5–4.5	27–29	22	3.5–5.0	24–26	39	1.0–3.0	24–26	35	0.5–2.5
25°	11–13	33	2.5–3.5	10–11	15	0.5–1.5	10	20	0.0–0.2	9–10	42	0.0
28°	10–11	26	0.5–1.2	9–10	19	0.0	8–9	29	0.0	8–9	34	0.0

* Normal developmental time on full food is 9 days (215 hours) from egg-laying.

Effect of Yeast Concentration and Temperature

In order to determine the most suitable conditions for the starvation effect, series with varying yeast concentrations were incubated at different temperatures, 18°, 25° and 28° C. The results are given in Table I. It was found that 0.5 per cent dry yeast at 25° C. was most

TABLE II

Influence of carbohydrates on the starvation effect. Basic medium: 0.5 per cent brewers' yeast in 1.5 per cent agar.

	Carbohydrates added (2 per cent concentration)			
	None	Starch	Sucrose	Glucose
Delay in days	2–3	2–5	1–4	1–4
Number of flies	9	62	67	45
Eye color	2.0–3.5	0.0–0.5	0.0–0.3	0.1–1.0

suitable, both for the intensity of the effect and for the developmental time required. Lower concentrations of yeast at this temperature gave somewhat stronger effects, but mortality was higher. The higher temperature, 28° C., speeded up development and greatly decreased the intensity of the starvation effect; i.e., pigment production did not take place on yeast concentrations over 0.5 per cent. At 25° C., 3 per cent yeast or more prevented the starvation effect, while pigment appeared on all concentrations up to and including 5 per cent yeast at 17° C. This effect of temperature may be due to a differential influence on

TABLE III

Influence of sucrose concentration on eye-color (starvation effect) and length of larval life. Basic medium: 0.5 per cent brewers' yeast in 1.5 per cent agar. (Figures in parenthesis indicate number of flies.)

Prolongation of larval life	Sucrose concentration (*per cent*)							
	0	0.05	0.1	0.3	0.5	1.0	2.0	4.0
days 1			4.0–4.5 (4)	1.5–2.0 (21)	1.0–2.5 (60)	0.3–1.5 (22)		
2	2.0–3.5 (2)	4.0 (2)	3.5 (17)	3.5 (19)	2.5–3.0 (18)	0.5–1.5 (21)	0.0–0.2 (11)	
3	2.0–3.5 (7)	3.5 (20)	3.0 (10)	3.0 (7)	2.0–3.0 (6)	0.0–1.5 (8)	0.0–0.6 (19)	
4	2.0–4.0 (17)	2.0–3.0 (5)	3.0 (5)	2.5 (2)			0.0–0.5 (5)	0.0 (2)
5	2.5–3.5 (2)	1.5 (1)					0.0–0.8 (5)	0.0 (20)
Total	2.0–4.0 (28)	1.5–4.0 (28)	3.0–4.5 (36)	1.5–3.5 (49)	1.0–3.0 (84)	0.0–1.5 (51)	0.0–0.8 (40)	0.0 (22)

larval activity (food intake) and on the rate of metabolic processes. The medium containing 0.5 per cent yeast was selected for standard starvation and used throughout further work. At 25° C. it consistently delayed larval development from 2 to 4 days and gave eye-color values of from 2.5 to 3.5. This is equivalent to a v^+ hormone production of 3.5 to 8.0 units per individual (Tatum and Beadle, 1938). Controls were made for each series of experiments, with similar results. These control starvation values are omitted from the tables in most cases.

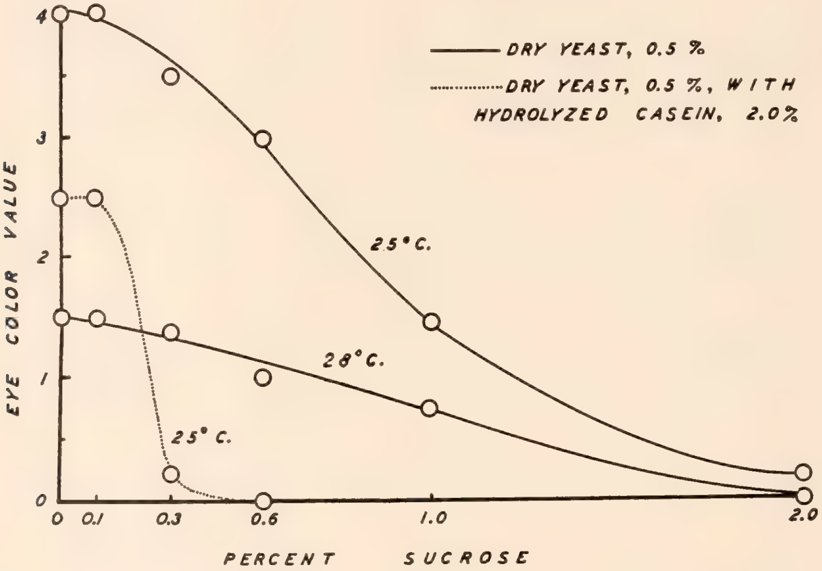

FIG. 1. Influence of temperature and of hydrolyzed casein on sucrose inhibition of the starvation effect (maximum color values for each sugar concentration used in plotting curves). Basic medium: 0.5 per cent brewers' yeast in 1.5 per cent agar.

Effect of Carbohydrates

Table II shows the influence of added carbohydrates on the starvation effect. Starch, sucrose, and glucose almost completely inhibited the production of pigment, although larval life was prolonged as much as or more than in the controls without carbohydrate. Several series of experiments were made to establish the relation of sugar concentration to prolongation of larval life and intensity of the starvation effect. Table III gives the combined results of these series. Concentrations of sucrose up to 0.5 per cent shortened larval life as compared to the control, without very marked effect on eye pigmentation. Higher sucrose concentrations progressively prolonged larval life and inhibited pigmentation. Two per cent sucrose caused about the same delay as in the control, but almost completely prevented v^+ hormone production. Four per cent sucrose seemed to be toxic and prolonged larval life even more than in the control, but completely suppressed the starvation effect.

The inhibiting effect of varying concentrations of sugar at 28° C. was also determined. The influence of temperature on the sucrose effect is shown in Fig. 1. It required almost the same concentration of sugar (2 per cent) to inhibit completely pigmentation at the higher temperature as at 25° C., although the production of pigment on the control starvation food without sugar was much less at 28° C.

TABLE IV

Influence of other substances on the starvation effect. Basic medium: 0.5 per cent brewers' yeast in 1.5 per cent agar.

Substance added	Prolongation of larval life in days	Number of adult flies	Eye color
Sodium benzoate, 1 per cent*	1–3	20	0.1–1.5
Sodium benzoate, 1 per cent†	2–3	5	0.5–0.8‡
Calcium acetate, 2 per cent	6–9	30	0.0–0.2
Calcium lactate, 3 per cent	5–9	30	1.0–3.0
Calcium carbonate, 2 per cent	6–9	20	2.0–3.5
Ethyl alcohol, 5 per cent§	1–5	22	0.0–0.1
Glycerol, 2 per cent	3–7	38	2.0–3.0
Butter fat, 4 per cent	3–8	11	0.0–0.2

* Sterile 60-hour-old fully fed larvae transferred aseptically to test medium.

† Eggs not sterilized.

‡ Control with no benzoate; color = 3.0.

§ Alcohol added after cooling medium to 35°C.

The results of these experiments with carbohydrates show that prolongation of larval life is not necessarily accompanied by v^+ hormone and eye pigment production. However, the starvation effect is observed only when larval life is prolonged.

Influence of Other Substances on the Starvation Effect

It seemed possible that some indication of the nature of the starvation effect might be obtained by similarly testing substances other than carbohydrates. Table IV summarizes the results of these experiments. Calcium lactate, calcium carbonate, and glycerol had only very slight inhibiting effects on pigmentation. On the other hand, ethyl alcohol, butter fat, and calcium acetate prevented the starvation effect almost completely. Sodium benzoate was quite toxic, but under non-lethal conditions it prevented pigment production to a considerable degree.

Each of these various additions to the starvation diet considerably prolonged larval life, but production of v^+ hormone was suppressed only by certain specific substances, all of which, with the exception of sodium benzoate, may be assumed to be metabolized in a manner similar to carbohydrates. No explanation can be suggested for the inability of glycerol and calcium lactate to function in this way. Concentrations of calcium lactate and glycerol from 0.5 to 3.0 per cent have been used with similar results in every concentration.

Effect of Proteins and Amino-acids

In contrast to carbohydrates, which definitely inhibit the starvation effect, whole and hydrolyzed proteins and mixtures of amino acids, including tryptophane, have no significant effect in reducing either duration of larval life or pigment production. These results are given in Table V.

TABLE V

Influence of protein and amino acids on starvation effect. Basic medium: 0.5 per cent brewers' yeast in 1.5 per cent agar.

Substance added	Prolongation of larval life in days	Number of adult flies	Eye color
Gelatine, 3 per cent	5–6	2	1.5–2.0
Gelatine, 3 per cent; Tryptophane, 1 per cent	5–9	7	1.5–2.5
Mixture of amino acids*	11	2	3.5
Hydrolyzed casein, 0.5 per cent	2–4	13	2.5–3.5
Hydrolyzed casein, 1 per cent	2–4	18	2.0–3.5
Hydrolyzed casein, 2 per cent	2–4	11	1.0–3.0
Hydrolyzed casein, 4 per cent	5–6	9	0.2–3.0

* Tryptophane, tyrosine, cystine, leucine, asparagine, glycine, alanine; 0.1 per cent each.

Although hydrolyzed casein alone had very little effect on pigmentation, it greatly intensified the sucrose effect. The result of a series containing 2 per cent hydrolyzed casein and increasing amounts of sugar is graphically represented in Fig. 1. In the presence of hydrolyzed casein, a sucrose concentration of 0.3 per cent almost completely inhibited hormone production. The other curves in Fig. 1 give for comparison the effect of sucrose without hydrolyzed casein. In the presence of an excess of amino acids, the sugar concentration effective in pigment inhibition was about that optimal for growth (see Table III).

Discussion

Khouvine et al. (1938) suggested that sugar may have a protein-sparing action, and that the starvation effect and production of v^+ hormone involves an abnormal protein degradation in the larva. It seems possible, from our results, that the action of carbohydrates and similar substances may be due to their protein-sparing action. However, it is probable that other factors are also involved since the sugar concentration optimal for growth does not inhibit the starvation effect and pigment production. This concentration (0. 5 per cent) should have the same protein-sparing action as higher concentrations. In the presence of an adequate supply of amino acids (hydrolyzed casein), however, sugar completely inhibits the starvation effect at the 0.5 per cent concentration optimal for growth.

Carbohydrates seem to inhibit pigment production in starvation by altering the starvation metabolism in such a way that v^+ hormone is not produced, and not by affecting the utilization of the hormone. Khouvine et al. showed that a diet containing sugar did not affect the utilization of ingested v^+ hormone supplied as a *Calliphora* extract. In addition, we have injected mixtures of glucose with extracts containing v^+ substance into *v bw* larvae with no decrease in the effectiveness of the hormone.

Substances other than carbohydrates which also prevent the starvation effect probably act in the same way, since theoretically they may be metabolized in a similar manner. The action of sodium benzoate, since it has no relationship to carbohydrates metabolically, may have a different basis. Sodium benzoate acts similarly to sugar in that it inhibits the production of v^+ hormone by *v bw* larvae on a starvation diet. However, it has no effect on the normal hormone production by *su²-v, v bw* larvae (normal eye-color ♂ = 1.0; ♀ = 2.0). Nor does sodium benzoate influence the utilization of ingested v^+ hormone by *v bw* larvae.

Beadle et al. (1938) showed that starvation is effective only during a certain sensitive period in larval development. This period was found to lie between 60 and 70 hours of normal development. The starvation effect may be assumed to be a result of prolonging this specific developmental period. Preliminary experiments designed to determine the effect of sucrose on this sensitive period were carried out under aseptic conditions by placing fully fed 54-hour-old larvae on low food with and without sugar. At intervals thereafter larvae were removed from the starvation food to plain agar. The ability to pupate served as the criterion of the end of the 60–70-hour sensitive period (see Beadle et al., 1938). The results seemed to indicate that this period is significantly shortened by sugar in the starvation food.

Whether the action of carbohydrates in inhibiting the starvation effect is due to a direct influence (possibly through a protein-sparing action) on specific processes which during the starvation period lead to the production of v^+ hormone, or whether it is due to a differential acceleration of development during the 60–70-hour sensitive period, thereby shortening the effective time of starvation, cannot be definitely decided at present.

SUMMARY

The production of v^+ eye-color hormone and development of pigment in the double recessive vermilion brown of *D. melanogaster* may be brought about by feeding the larvae on sub-optimal levels of dead yeast under aseptic conditions.

With a given concentration of yeast, culture of larvae at low temperature (17° C.) greatly increases the intensity of the starvation effect. High temperature (28° C.), on the other hand, decreases the intensity of the starvation effect.

Carbohydrates and related substances (acetate, fat, and ethyl alcohol) added to the low yeast diet, under aseptic conditions, completely inhibit the starvation effect by their direct action on larval metabolism and development.

Proteins and amino-acids have very little influence on the starvation effect, but greatly lower the carbohydrate level required to completely inhibit pigment production.

The starvation effect is always associated with prolongation of larval life, but great prolongation of life is possible under certain conditions without any modification of eye color.

The inhibition by carbohydrates may be due to a direct influence on processes proceeding during starvation or to a specific acceleration of development during the period sensitive to starvation, or to both.

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PIGMENT INHERITANCE IN THE FUNDULUS-SCOMBER HYBRID

ALICE RUSSELL

(*From the Marine Biological Laboratory, Woods Hole, Mass.*)

The hybrid between *Fundulus heteroclitus* (L.) ♀ and *Scomber scombrus* (L.) ♂ is first mentioned by H. H. Newman in 1915. J. Loeb had stated his belief that development in intergenic hybrids is parthenogenetic. Newman cites the inheritance of *Scomber* pigmentation in the *Fundulus-Scomber* cross as a proof that fertilization had taken place. In a later paper, 1918, he continued the discussion of this hybrid.

His account of the abnormalities found in the embryos made it seem worthwhile to obtain the cross again, to make a cytological and morphological study of early stages, and to make a detailed study of the chromatophores in parent and hybrid embryos.

Preliminary hybridizations were made successfully late in June, 1937, at the Marine Biological Laboratory. During the summers of 1938 and 1939 numerous hybridizations were made from June 10–July 5. After July 5 it is usually impossible to procure spawning mackerel, before June 10 it is difficult to obtain spawning *Fundulus*. For best results, with a large percentage of hybrid embryos, both parents must be at the height of sexual activity.

Ripe *F. heteroclitus* females were selected and isolated in tanks of running sea water for at least 18 hours before they were to be used: this assures the absence of *Fundulus* sperm. As mackerel do not live long after being caught, hybridizations were carried out at the traps. *Fundulus* females were carried to the fish traps in clean buckets or bowls of sea water. *Scomber scombrus* males were stripped into finger bowls containing a small quantity of sea water. The *F. heteroclitus* females were stripped into the sperm suspension, the eggs from each female being kept in separate bowls. After 10–15 minutes the sperm suspension was washed off and fresh sea water was added. In the laboratory the eggs were placed a few in each bowl, and allowed to develop. Controls were carried as follows: (1) Unfertilized *F. heteroclitus* eggs from some of the females in each set of hybridizations were observed as a check on the possible presence of *F. heteroclitus* sperm in sea water, in the tanks, or on the fish. (2) *F. heteroclitus* eggs were fertilized with *F. heteroclitus* sperm to check on the fertilizability and rate of development of normal *Fundulus heteroclitus.* (3) *Scomber scombrus* eggs were fertilized with *Scomber scombrus* sperm to check on the normal *Scomber scombrus* development. (4) The reciprocal cross with *Scomber scombrus* eggs and *F. heteroclitus* sperm was tried many times, always unsuccessfully.

The egg of *Fundulus heteroclitus* is 2–2.5 mm. in diameter, well yolked, demersal, developing slowly and hatching out in 12–16 days. The egg of *Scomber scombrus* is smaller, 1 mm. or less in diameter, transparent, pelagic, developing quickly and hatching in 60–72 hours. The hybrid develops more slowly than normal *F. heteroclitus,* forming defective embryos which, in our experience, never hatch, even though kept for 30–35 days.

Normal stages of *Scomber scombrus* have not been described, although Worley (1933) mentions that they resemble closely those of sea bass as described by Wilson (1889). As there was no published account of pigment development in *Scomber scombrus,* this had to be studied. *Scomber scombrus* eggs were obtained and fertilized at the fish traps. As the eggs are pelagic, it is difficult to wash off the excess milt while in transit from traps to laboratory; sea water can, however, be added from time to time. *Scomber* eggs are very sensitive to temperatures above 17° C., and will not develop at all above 21° C. (Worley, 1933), therefore care must be taken that the water in the shallow bowls is not warmed by the sunshine, or by heat from the decks or engine. In the laboratory the fertilized and developing eggs soon float to the top of the water, and can be skimmed off and transferred to fresh sea water. If the bowls are placed in baths of running sea water the eggs develop quite normally, and hatch out in 60–72 hours. The first pigment cells to appear are the slender branching melanophores on the dorsal surface of the embryo at 27 hours. Later more melanophores appear and form the characteristic pattern; a band across the dorsal surface of the head at the level of the optic vesicles, and a row along the lateral line region. A few migrate to the yolk sac and to the oil drop. At 36 hours there appears just behind the optic vesicles a group of cells containing yellowish green pigment granules. Soon the granules increase in number, the pigment cells fuse, forming two large brilliantly green chromatophores persisting at least as long as the fry live in the laboratory. Other green chromatophores may appear behind the otic vesicles, on the oil drop or near Kupfer's vesicle. Upon hatching the young fry drop to the bottom of the vessel and lie there until they are able to swim about easily.

Normal stages of *Fundulus heteroclitus* have been described (Oppenheimer, 1937). Typical pigment formation has been described, also

(Bancroft, Stockard, Newman). Four days after fertilization a first head crop of melanophores appears. Another crop appears on the fifth day. The pigment cells are of three types: those on the yolk are large polygonal melanophores with but few processes, those on the embryo are smaller and more branched: the reddish-orange much-branched chromatophores found on embryo and yolk sac. On the sixth day, when circulation begins, the melanophores of the yolk sac migrate to the blood vessels and fuse. The reddish-orange chromatophores also arrange themselves along the course of the blood vessels, but do not seem to fuse. A number migrate to the lateral line. Melanophores are rarely seen in this region, the absence of a visible lateral line being one of the species characteristics of the adult *F. heteroclitus.* After hatching few reddish chromatophores are to be found on the exterior of *F. heteroclitus.*

The melanophores of *F. heteroclitus* and *S. scombrus,* the green chromatophores of *Scomber,* and the reddish chromatophores of *Fundulus* all contain a granular pigment. No green chromatophores are ever present in *Fundulus heteroclitus,* and no red chromatophores in *Scomber scombrus.*

Fundulus-Scomber hybrids cleave at the same rate, or more slowly, than normal *Fundulus heteroclitus.* In a series of 15 hybridizations, 3,097 eggs were fertilized: 3,084, or 99 per cent of these cleaved. Many died at gastrulation and during early embryonic life, but 1,205 or 39 per cent formed advanced embryos. Development in the hybrid is slower than in normal *F. heteroclitus:* pigmentation develops later, the heart does not begin to pulsate as early, circulation is feeble, or not established in most of the hybrids. As a result, the chromatophores remain scattered for a longer period, eventually migrating to the heart, or to the site of its attachment to the yolk. As has been noted by Bancroft, Newman and others, the hybrid embryos show various combinations of the parental types of chromatophores.

Figure 1 *a* shows the average melanophore counts for parent and hybrid embryos. As their rate of development differs widely stages which are equivalent were arbitrarily chosen. Normal *F. heteroclitus* five days after fertilization shows both first and second crops of melanophores not as yet fused on the blood vessels. *Scomber scombrus* at 30 hours shows first and second crops of melanophores also, not yet migrated to head and lateral line regions; the hybrids at 7 days showed the melanophores well developed, not yet fused. *F. heteroclitus* embryos show from 8–18 melanophores, or an average of 13—on the dorsal surface; *S. scombrus* has from 34–51, averaging 40. The *Fundulus-Scomber* hybrid shows a great variation, from 2–44. The majority of

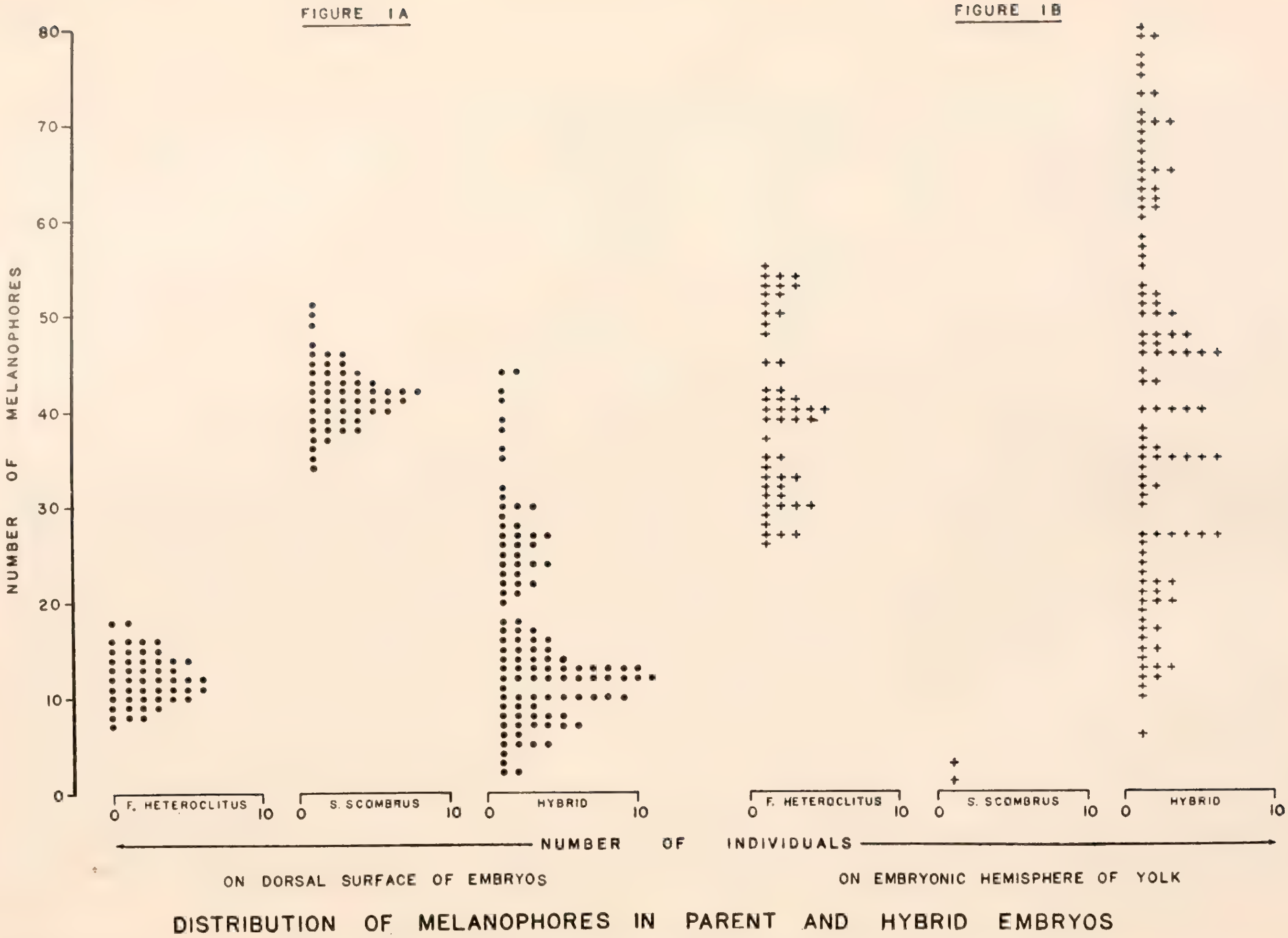

FIG. 1. *A* and *B*. Distribution of melanophores in parent and hybrid embryos.

hybrid embryos seem to show a tendency to conform to the *F. heteroclitus* type of distribution, having 8–18 melanophores on the dorsal surface of the embryos. However, we have seen no normal *F. heteroclitus* embryos of 5 days with over 20 melanophores—the increase in number of melanophores present in a large number of hybrid embryos may be assumed to be the influence of *Scomber scombrus.*

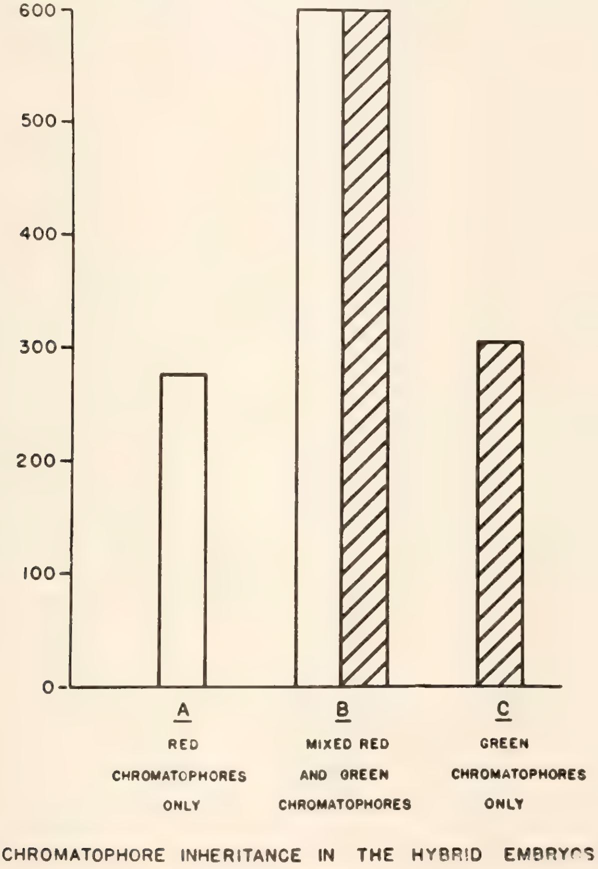

FIG. 2. Chromatophore inheritance in the hybrid embryos.

Figure 1 *b* shows the distribution of yolk melanophores, counted on the embryonic hemisphere of the same individuals used above. In *F. heteroclitus* the yolk melanophores are large polygonal cells, varying from 26–55 in 5-day-old embryos. *Scomber scombrus* at 30 hours rarely shows any yolk melanophores. In the 7-day-old hybrid embryos the number varies from 6–80, with scarcely two embryos having identical numbers of melanophores.

The hybrids can be grouped roughly into three categories: those showing green chromatophores of the *Scomber* type, but no red *F. heteroclitus* type chromatophores: those showing the red *Fundulus* type, but no green *Scomber* type chromatophores: those showing both red and green chromatophores. Figure 2 will show the distribution of 1,205 ten-day-old hybrid embryos in these three categories. There seems to be a very significant relation at first glance, since about equal numbers of individuals show only maternal or paternal type chromatophores, and about twice as many show both types combined. As a matter of fact, examination shows that no two embryos are identical. In the group showing green chromatophores only, the individuals range from those brilliantly green laterally to some with only a few green head chromatophores: some show green chromatophores on the embryo only, some show them on the yolk also. In the group showing only red chromatophores of *Fundulus* type, the individuals are equally variable, some embryos being brilliantly reddish-orange, others grading to some resembling closely *F. heteroclitus* embryos. In the large group showing both red and green chromatophores there is every conceivable type of combination, no two individuals are identical. In addition, the *Scomber* and *Fundulus* types of melanophores are present in all possible combinations with the red and green chromatophores, in all three categories. No consistency of pigment distribution is found in sets of *Fundulus* eggs from various females, fertilized at the same time by sperm from the same mackerel: some may show a preponderance of green, of red, or of mixed green and red chromatophores as will be seen in Table I.

TABLE I

Green Chromatophores Only	Mixed Red and Green	Red Only
84	57	43
27	41	3
27	107	35
2	29	21
2	12	29

These counts were made on eggs from various *F. heteroclitus* fertilized by sperm from the same mackerel on June 17, 1939, and counted June 27, 1939.

Unlike Newman, who reports that green chromatophores can be found only in hybrid embryos obtained before mid June, we have found green chromatophores in the hybrids whenever we got successful crosses.

DISCUSSION

Many intergenic and interspecific crosses have been described. Some of these "hybrids" do not develop beyond late embryological stages, as is true of the *Fundulus-Scomber* hybrid we are discussing. Here we are dealing with a much wider cross, for the two genera belong to different sub-orders. They differ widely in ecological relations, in habitat, and in structure. Morphological studies of hybrid and parent embryos may show significant combinations of structural peculiarities, for example: *F. heteroclitus* has an air bladder, while this structure is absent in *Scomber scombrus*. Cytological studies may show successive elimination of chromosomes during early cleavages. It is probable that some of the embryos showing only *Fundulus* type chromatophores, and typical *Fundulus* distribution of melanophores, may be haploid individuals. These individuals are rare, however, for even in this group one generally finds some *Scomber* type melanophores, or some *Scomber* effect on the number or distribution of melanophores. (Figure 1, *a* and *b*.)

It is probable that in the teleosts, as in the amphibians, pigment differentiation depends on neural crest development. If so, it is reasonable to suppose that wherever *Scomber* type chromatophores are present, *Scomber* chromosomes may have been retained throughout cleavage, gastrulation and differentiation.

We have purposely refrained from a discussion of the size of melanophores and chromatophores in parents and hybrids, for any measurements would be open to the criticism that metabolic processes in the embryo are abnormal, or at least, much disturbed. In shape, the melanophores of *Fundulus* are quite distinct and distinguishable (Newman and others). In color the reddish chromatophores in the hybrids are identical with those of *Fundulus heteroclitus,* the green chromatophores identical with those of the *Scomber* parent.

In the literature on interspecific and intergenic crosses, the F_1 generation is generally reported as intermediate. A closer scrutiny of the hybrids may reveal a much wider variation than hitherto suspected, as, for instance, in the case of the melanotic hybrids between *Platypoecilius* ♀ and *Xiphophorus* ♂ described by Gordon. Apparently a case of Mendelian dominance is revealed in the universally melanotic F_1 generation, but there is reported a variation in degree of melanosis in the progeny.

Earlier reports (Newman, Bancroft) have attempted to explain the results of the *Fundulus-Scomber* hybridizations on the basis of Mendelian dominants and recessives, or of " blending " inheritance. However, as no detailed study of pigmentation was attempted, the enormous variability actually present in the hybrids escaped attention.

Pinney, reporting on other inter-sub-order hybrids, reports chromosome elimination during early cleavages.

We have at this time no satisfactory explanation for the phenomenon presented by the pigment inheritance in this cross, although we may assume that there is a complicated random assortment, combination and elimination of chromosomes.

We are indebted to Mr. Robert Goffin of the U. S. Bureau of Fisheries, at Woods Hole, and to the crew of the " Sagitta " of the Marine Biological Laboratory for their assistance in getting to the fish traps and obtaining mackerel. We wish also to thank Dr. H. B. Goodrich, for his interest, and also Rev. F. W. Ludwig, Ph.D., for help with the microphotographs.

Summary

1. Methods for hybridizing *Fundulus heteroclitus* and *Scomber scombrus* are described.

2. The pigment development in hybrid and parents is described.

3. Comparison of the inheritance of embryo and yolk melanophores reveals a *Scomber*-effect in the embryo.

4. The hybrid embryo shows chromatophore inheritance from both parents.

5. Actually, as regards inheritance of melanophores and chromatophores, there is enormous variability, no two embryos being identical as to pigment distribution.

6. This variation in the F_1 generations is unusual and at present inexplicable.

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THE EFFECT OF INCREASING TIME OF DEVELOPMENT AT CONSTANT TEMPERATURE ON THE WING SIZE OF VESTIGIAL OF DROSOPHILA MELANOGASTER

GEORGE CHILD

(*From the Department of Biology, Amherst College, Amherst, Massachusetts*)

INTRODUCTION

This paper deals with the effect of nipagin (methyl parahydroxy benzoate) and poor food conditions on the wing size of the mutant vestigial in *D. melanogaster*. Nipagin is being used in many laboratories as an antiseptic for mold control in *Drosophila* culture media. It was found at this laboratory that the time of development of an isogenic stock was increased when the larvae were raised on nipagin-treated food. This suggested a method for increasing the time of development at constant temperature, a new tool in phenogenetic research.

The effect of temperature on the wing size of vestigial has been studied by a number of investigators (Harnly, 1930, 1932; Stanley, 1928, 1931, 1935; Hersh and Ward, 1932; Li and Tsui, 1936). They find that the wing size of vestigial increases with increasing temperature. Furthermore, the temperature-effective period occurs during a relatively short portion of the larval life. This present work represents a different approach to the problem in that it concerns the effect of prolonging the duration of the larval period at constant temperature.

METHODS

The culture medium consisted of 850 cc. water, 100 grams cornmeal, 150 cc. molasses, 13 grams agar-agar, and 6 grams brewer's yeast, made up in the usual manner. The cultures were seeded with dry yeast. In the starvation experiments the dry yeast was not added. The nipagin was weighed on a standard quantitative balance and thoroughly mixed with the food before pouring. Half-pint milk bottles containing 60 cc. food were used.

The isogenic vestigial stock was obtained from Dr. A. Hersh of Western Reserve. About 20 pairs were used for egg-laying. It was found that the flies did not lay well on the food containing the higher concentrations of nipagin. It was necessary to use long egg-laying periods of twelve hours and for this reason the time of development was determined only approximately for these concentrations.

The egg-laying and total development was carried out in an incubator held constant at 28° ± 0.1°. The incubator was kept in a constant temperature (16° ± 1.°), constant humidity (60 per cent ± 5 per cent relative humidity) room. The apparatus is fully described in Drosophila Information Service 6, April 1936.

As the flies hatched they were examined, the sexes were separated and they were placed in vials containing 70 per cent alcohol. An unselected sample of control flies and 0.2 per cent nipagin flies were bred for a second generation to determine any "carry-over" effect. The wings of the flies were removed under a binocular microscope and mounted on slides with a drop of cedar oil. The right wing was used unless it was torn or mutilated.

TABLE I

Effect of nipagin on wing size of vestigial. Temperature, 28° ±0.1°.

Conc. of Nipagin in per cent	Time of Pupation (hours)	♀♀			♂♂		
		No.	Length in mm. ±s.e.	Area in mm.^{2} ±s.e.	No.	Length in mm. ±s.e.	Area in mm.^{2} ±s.e.
0.0	98	40	0.85 ±0.017	0.178 ±0.0047	14	0.76 ±0.023	0.132 ±0.0033
0.05	104	44	0.90 ±0.007	0.186 ±0.0043	25	0.91 ±0.035	0.177 ±0.0078
0.1	112	37	0.90 ±0.015	0.171 ±0.0044	41	0.95 ±0.024	0.189 ±0.0072
0.2	146	50	1.02 ±0.003	0.213 ±0.0094	45	1.06 ±0.035	0.221 ±0.0094
Carry-over effect F_1 from random sample of .2 per cent nipagin-treated flies							
0.00	103	31	0.95 ±0.023	0.206 ±0.0055	38	0.85 ±0.0075	0.167 ±0.0038

The wings were projected with a Proni projection apparatus. The magnification set at 75 × was checked periodically with a stage micrometer. The periphery of the wings were traced and from these tracings the maximum lengths and areas were measured. A Glogau vernier caliper and a Keuffel and Esser planimeter were used.

In the later experiments 0.1 per cent nipagin was used. It was suspected that nipagin produced its effect by slowing down the growth of yeast and thereby decreasing the food supply. To check this a number of cultures were prepared with no addition of live yeast. In these nonseeded bottles a number of old larvae were added after the egg-laying. This was done to remove any yeast carried in on the bodies of the adult flies.

The time of development was determined by removing pupae at intervals of four hours and placing them on agar slants. Many of these flies were used for a second generation test. The matings were control × control, control ♂♂ × nipagin-treated ♀♀, control ♀♀ × nipagin-treated ♂♂ etc. to determine how much of the carry-over effect was maternal or paternal.

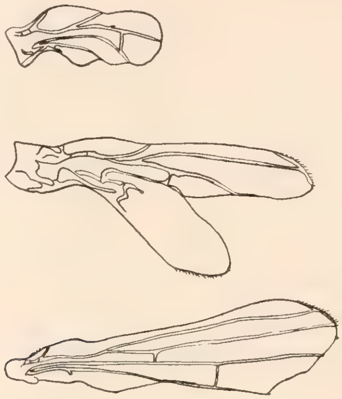

Fig. 1. A normal 28° vestigial wing compared with wings from 28° nipagin treated flies.

Experimental

The Effect of Varying Concentrations of Nipagin

In these experiments the larvae were raised on nipagin-treated food to determine the effect of nipagin on the time of development and wing area. The concentrations of nipagin used were 0.05 per cent, 0.1 per cent, 0.2 per cent, 0.4 per cent, and 0.8 per cent of the food weight. A few larvae in the 0.4 per cent and 0.8 per cent developed to pupation but failed to hatch. It was found that the time of development increased with increasing concentrations of nipagin, the 0.2 per cent pupating two days later than the controls.

Table I shows the wing length and area as affected by nipagin. With one exception, the 0.1 per cent for females, the lengths and areas of the vestigial wings increase with increasing concentrations of nipagin. The wing size of the control males is smaller than that of the females. With increasing concentrations of nipagin, however, the male wing size increases faster than the female. At 0.1 per cent and 0.2 per cent the male wings are larger than the female. A similar result is obtained with temperature, the male wing size exceeding that of the females at high temperature (Harnly, 1930; Stanley, 1931).

It was found when preserving the flies at hatching, that with increasing time of development there was an apparent increase in wing size within each nipagin-treated population. The change in wing size due to nipagin is, therefore, greater than the means given in Table I; these means having been obtained by including all the flies in a given population irrespective of their time of development. This general result has been recently reported by Braun (1939) on notch.

TABLE II

Relation between time of pupation and wing area. Control series.

Time of Pupation in hours	♂♂		♀♀	
	No.	Area in mm.	No.	Area in mm.
1. 82	8	0.130	3	0.164
2. 86	6	0.138	6	0.137
3. 90	15	0.137	6	0.159
4. 94	25	0.136	10	0.143
5. 98	7	0.154	11	0.173
6. 102	3	0.158	4	0.147
7. 106	3	0.133	2	0.187
8. 110	0		2	0.167
9. 114	3	0.107	2	0.154
mean time, 93.2±0.87 hours mean area, 0.139±0.004 sq. mm.			mean time, 96.1±1.24 hours mean area, 0.156±0.0035 sq. mm.	

Figure 1 illustrates the appearance of the larger wings obtained from a 0.2 per cent nipagin population as compared with a "normal" vestigial wing. The larger wings simulate the expression of other vg. alleles when raised under normal environmental conditions.

The results obtained by raising a random sample of 0.2 per cent nipagin-treated flies for another generation but in untreated food are shown in Table I. Both the mean length and area show a significant carry-over effect. Some of these data had been reported previously (Child and Albertowicz, 1936).

Effect of Time of Development

In the second series of experiments the relation between time of development and wing area was determined. Starvation and 0.1 per cent nipagin were used. The larvae were removed from the culture as they pupated and the areas of the wings were determined separately for each pupating group. The results (Table II) indicate that in the control series there is no apparent effect of time of development (from egg-laying to pupation) on the size of the wings. The larvae pupate between 82 hours and 114 hours and the wing areas among the different groups do not differ significantly from one another.

The time of development is very markedly increased in the nipagin and starvation series (Tables III and IV). The wing areas of the various groups show greater differences than in the control series. There is an apparent relation between the time of pupation and wing area. This relation is more easily observed on the imagoes as they hatch. With increasing time of development the larvae (and the flies) become smaller and smaller so that the relative difference between wing area and body size is very great in the delayed flies although the absolute area increases and then decreases. Unfortunately the body size of the adults was not measured and we are unable to show this difference quantitatively.

TABLE III

Relation between time of pupation and wing area after treatment with 0.1 per cent nipagin.

Time of Pupation in hours	♂♂		♀♀	
	No.	Area in mm.	No.	Area in mm.
1. 93	10	0.158	12	0.167
2. 97	8	0.231	5	0.181
3. 101	2	0.437	4	0.140
4. 107	23	0.299	12	0.172
5. 118	9	0.368	8	0.181
6. 129	5	0.592	5	0.236
7. 141	6	0.517	1	0.268
8. 153	1	0.505	3	0.233
9. 165	25	0.268	8	0.151
10. 179	23	0.267	23	0.165
11. 191	7	0.318	14	0.208
12. 203	12	0.379	14	0.190
13. 215	14	0.354	27	0.201
mean time, 152.4±3.39 hours mean area, 0.315±0.0113 sq. mm.			mean time, 163.5±3.81 hours mean area, 0.185±0.0044 sq. mm.	

The Carry-over Effect

The carry-over effect was studied using normal food. To determine whether both sperm and eggs from treated parents transmitted the fac-

TABLE IV

Relation between time of pupation and wing area after starvation.

Time of Pupation in hours	♂♂		♀♀	
	No.	Area in mm.	No.	Area in mm.
90	7	.204	5	0.190
94	3	.164	3	.310
98	2	.197	12	.185
102	5	.194	2	.238
106	1	.224	4	.188
110	2	.169	7	.169
116	17	.277	16	.201
122	4	.258	8	.196
126	4	.283	4	.215
130	3	.212	4	.100
138	6	.242	9	.300
150	0		3	.231
162	1	.345	1	.212
174	2	.642		
mean time, 116.4±2.53 hours mean area, 0.254±0.0174 sq. mm.			mean time, 115.0±2.13 hours mean area, 0.213±0.0102 sq. mm.	

tors for increased wing size, treated males and females were mated with control females and males respectively. Treated males were also mated with treated females. The results of these various reciprocal crosses are shown in Table V which also includes the control areas and the means of Tables III and IV. The carry-over effects are more apparent

TABLE V

Effect of .1 per cent nipagin and starvation.

Experiment	♂♂			♀♀		
	Time of Pupation	No.	Area ±s.e.	Time of Pupation	No.	Area ±s.e.
	hours		*mm.*^{2}	*hours*		*mm.*^{2}
Control	93.2±0.87	70	0.139±.0040	96.1±1.24	48	0.156±.0035
.1% Nipagin	152.4±3.39	145	0.315±.0113	163.5±3.81	136	0.185±.0044
Starved	116.4±2.53	57	0.254±.0174	115.0±2.13	78	0.213±.0102
Previous Treatment	Carry-over effect *F*$_1$ from control and treated flies					
Control ♀ × Nipagin ♂	110	94	0.203±.0058	110	81	0.181±.0019
Control ♀ × Starved ♂	110	33	0.203±.0163	110	49	0.183±.0025
Nipagin ♀ × Control ♂	116	25	0.229±.0270	116	36	0.201±.0167
Starved ♀ × Control ♂	116	45	0.281±.0203	116	56	0.192±.0060
Nipagin ♀ × Nipagin ♂	116	40	0.279±.0215	116	44	0.174±.0042
Starved ♀ × Nipagin ♂	116	55	0.356±.0259	116	31	0.224±.0141
Control ♀ × Control ♂	95	22	0.143±.0052	95	19	0.161±.0037

in the male offspring than in the female offspring, since in the latter the total effect is smaller. The time of development is only approximate, not having been measured by pupa removal but by simply noting the time when about half the larvae had pupated. It is quite evident that in all of the matings the wing areas are greater when affected flies of either sex are used as parents. When treated females are used as parents the difference is greater than when males are used. Treated males and females as parents have offspring with greater wing areas than those obtained when only one treated parent is used. Starvation of the parents seems to produce a greater effect in the offspring than nipagin treatment.

DISCUSSION

It is well known that with increasing temperature there is an increase in the wing size of vestigial. A sharp increase is not obtained, however, until very high temperatures are reached. It is generally accepted that temperature produces its effect by affecting differentially the rate or duration of the " vestigial reaction " as compared with the rate or duration of other developmental processes. By vestigial reaction we mean the developmental reaction or reactions in the vestigial fly which differ in rate or duration from the reactions in their isogenic wild type.

In the experiments with nipagin there is little reason to suspect that the change in wing size is due to a direct effect of nipagin on the vestigial reaction. The evidence, moreover, indicates that nipagin produces its effect by increasing the time of development. The temperature-effective period of the vestigial reaction is known to occur during a portion of the larval development. Thus, by increasing the larval period at constant temperature an effect on the vestigial wings will be produced if the duration of the vestigial reaction as compared with the rest of development is differentially affected. It appears from these results that such is the case.

In the first experiments, using varying concentrations of nipagin, it appeared that this chemical increased the time of development by decreasing the food supply. The yeast did not grow very well in the treated food although all bottles started with equal amounts of dead brewer's yeast and live yeast. The starvation experiments showed that this was the case. There was a definite increase in the time of development in wing area under both types of environmental conditions.

As stated previously, the exact relation between time of development and wing area is obscured because of the decrease in the size of the fly with increasing time of development. Under normal conditions the larvae begin to pupate at 82 hours and the last larvae pupate at 114 hours in these experiments. This variation is great because of the four-hour egg-laying periods but with even shorter egg-laying periods a spread of 18–24 hours is obtained (Powsner, 1935; Child, 1935).

This variation is a direct corollary to the nature of development which as Wright (1934) points out is the result of a large number of physical and chemical reactions, the rates and durations of which are determined by the history of the organism prior to the stage in question, correlative reactions within the organism, external environmental factors, actions of the genes within each cell, etc. In the highly heterogeneous systems of a developing larva these reactions will not go on at exactly the same rates in all organisms and there will of necessity be a spread in time of development as well as wing area under normal conditions *but no correlation between these measurements.* However, when an additional factor, lack of food, is superimposed upon this normal variation a new set of conditions prevails. The duration of the larval (feeding stage) period is lengthened, various reactions may produce minimal or even subminimal concentrations for further development and development will become somewhat disorganized. In other words, there will be a differential effect on the rates and durations of many embryological processes resulting in a modified phenotype. Under such conditions there will be a definite correlation between time of development and wing area.

With this general hypothesis in mind it is possible to postulate a number of mechanisms to account for the increased wing size. A simple scheme would allow the wing formation reactions to proceed at their normal rate but the developmental reactions which normally parallel them are slowed down, especially those reactions which determine the time at which the wing development stops. This would permit of an increased wing area. With further starvation even the wing reactions are slowed down or produce subminimal concentrations and the size of the wing decreases. This outline is, of course, very general and is not the only one which can be postulated. It merely illustrates how the general theory can be utilized.

Carry-over Effect

The carry-over effect experiments were unfortunately not extended beyond the first generation. They show, however, that there is a definite effect on the offspring of parents raised under poor food conditions—a sort of dauermodification (Jollos, 1934). It is well known that starved flies lay smaller eggs than normal ones. Powsner (1935) found that eggs laid by flies raised on poor food had a longer developmental period than eggs laid by flies raised on good food. If this delay

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in development were the only factor involved a definite carry-over effect should be expected on the maternal side. In these experiments, however, there was also a paternal effect. To account for this result one must assume an effect of starvation on developing sperm. This may concern the small amount of cytoplasm carried by the sperm or perhaps a direct effect on its genic material.

The recent series of investigations at Columbia University by Rittenberg, Schoenheimer, Clarke and others in which deuterium, isotopes of nitrogen, and other elements were used to follow intermediary metabolism may bear on this problem. These workers have shown that many of the organic substances in protoplasm, even proteins, are not in a static condition. The " living proteins " are constantly interacting with their environment and may exchange their hydrogen for deuterium, and even nitrogen for one of its isotopes. Thus the composition and behavior of protoplasm is directly modified by the composition of its environment. Should the chromosomes or the genes behave in this kinetic manner of extracting substrates from the cytoplasm and releasing equivalent substances in exchange, we would have a mechanism for the production of these starvation effects and other dauermodifications, production of immunity, even differentiation during ontogeny. It is necessary, of course, to assume that the cytoplasm of the treated flies differs from that of normal cytoplasm. In this manner a modified cytoplasm may produce a change in the chromosomes. It is also of interest to note that if this is the case, we have a mechanism for an " inheritance of acquired characters," not in the old sense of the phrase but on a molecular level. This would allow the environment to produce " genetic changes " which need not be permanent. These " mutations " could return to normal in one or more generations. Plough and Ives (1935) found that variations continued to appear in generations later than those actually treated with a high temperature of 36.5° for 24 hours. These variations decreased in number in subsequent generations.

These experiments are to be continued for a number of generations. The original vestigial stock used has been discontinued in this laboratory and another isogenic stock is being prepared.

SUMMARY

The time of development of an isogenic vestigial stock of *D. melanogaster* was increased by two methods: (1) by adding nipagin (ethyl parahydroxy benzoate) to the food, and (2) by adding only very little yeast to the food. Both methods are essentially the same in that the developing larvae are under starvation conditions. With increasing

concentration 0.05, 0.1, 0.2 and 0.4 per cent, there was an increase in the time of development and increase in the size of the wings, males showing a greater effect than females. The large wings resembled those of other vestigial alleles raised under normal conditions.

In another series of experiments 0.1 per cent nipagin and starvation were used. The larvae were removed from the culture as they pupated, to determine the relation between time of pupation and wing size. The first flies to pupate did not differ significantly in wing size from controls at that temperature. With increasing time of development there was an increase in wing size. Larvae which were very much delayed, however, developed into small flies with small wings. These wings, although small, were more differentiated and larger than the control wings.

The "carry-over" effect was studied using normal food. The treated females and males were mated with control males and females respectively. Treated males were also mated with treated females. The wings of flies from the latter mating showed the greatest carry-over effect. Treated females by control males resulted in flies having a significantly larger wing size than flies from the reciprocal cross. These results indicate that there is a definite effect on the germ cells of flies raised under starvation conditions, which effect shows itself in the subsequent development of the zygote.

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THE METHOD OF FEEDING OF TUNICATES

G. E. MacGINITIE

(From the William G. Kerckhoff Marine Laboratory of the California Institute of Technology)

Introduction

This, the second of a series of papers (MacGinitie, 1939) on the feeding mechanisms of marine invertebrates, deals with the method of feeding in three tunicates, namely, *Ciona intestinalis* (Linn.), *Ascidia californica* Ritter and Forsyth, and *Diplosoma pizoni* Ritter and Forsyth. The first two are simple ascidians and the latter is a colonial form. Since both simple and colonial forms have been investigated, I feel that it is fairly safe to state that the method described below is typical of all ascidians.

Young specimens of *Ciona intestinalis* and of *Ascidia californica,* especially those which have been reared in the laboratory, are quite transparent, and the observations here recorded were made upon animals which were in no way disturbed while they were carrying on their natural feeding activities. The same can be said of *Diplosoma pizoni,* as the matrix of the colony is perfectly clear and transparent. All observations were made upon undisturbed individuals of the colony.

Mechanism for Feeding

The structures which are strictly connected with the feeding activities of tunicates are the endostyle with its mucous glands, the peripharyngeal grooves, the dorsal groove, the stigmata, the esophagus, and the cilia lining all grooves, bars and the inner edges of the stigmata. The pharynx or branchial basket has been too well described in textbooks of zoology to make it necessary to redescribe it here. A current of water is maintained through the branchial cavity almost continually, whether the animal is feeding or not. The only time that the current is stopped is when the animals are left exposed by the tide or when they have been disturbed by some outside stimulus, and at such times the oral aperture and atriopore are usually closed.

The cilia lining the stigmata and the branchial basket may be divided into two groups, each group having a particular function. Those lining the stigmata have the function of maintaining the current of water, while those on the inner surface of the branchial bars and in the endostyle, the peripharyngeal grooves and dorsal groove have the function of moving mucus.

Food and the Method of Feeding

The endostyle is richly supplied with mucous glands, and when a tunicate starts to feed it begins to secrete mucus throughout the length of the endostyle. This mucus is moved by the cilia of the branchial bars around the branchial basket in two sheets, one on either side. When the edges of the mucous sheets arrive at the dorsal groove, they are taken up by it and formed into a thread, and this string is passed posteriorly along the dorsal groove to the esophagus. The function of the peripharyngeal grooves is to hold and move the oral ends of the two mucous sheets.

The water entering the branchial basket through the oral funnel passes into the atrial cavity through the stigmata in all directions with the exception of the region of the endostyle and dorsal groove, and when the animal is feeding such water must also pass through the sheet of mucus which covers the interior of the basket. This mucus intercepts and entangles all solid material entering with the water, and such material comprises the food of tunicates. On rocky shores it consists almost entirely of plankton, often greatly enriched by algal spores from seaweeds. Within the estuaries it consists largely of material in suspension, mainly stirred-up detritus from the shores and bottom. During the summer season in Southern California this detritus in suspension is usually enriched by one or more species of dinoflagellates.

While a tunicate is feeding mucus is constantly being secreted, and the mucous sheets covering the inner walls of the branchial basket move continuously from the endostyle toward the dorsal groove. Hence, while the tunicate is feeding, the food-laden thread of mucus enters the esophagus in an unbroken string. As it enters the stomach this mucous string is folded back and forth and remains intact for some time. It is only that portion near the pyloric valve that coalesces and becomes semiliquid as it passes into the intestine.

Although the cilia of the stigmata and branchial basket beat almost continuously, the mucous sheets are formed discontinuously. Upon the least disturbance the animals will cut off the secretion at the endostyle, and the remnants of the mucous sheets will continue to pass around to the dorsal side until the ends reach the dorsal groove. From then on until the animal begins to feed again the branchial basket is practically free of mucus. When a tunicate is not feeding, small particles may be seen to pass readily through the stigmata into the atrium and out with the atrial current.

If material which is foreign to the usual run of food material is introduced into the current of water entering the oral funnel, feeding will cease at once, and the undesirable material will be quickly forced from the branchial basket by a quick contraction of the body wall. The current will be renewed immediately, and, if no further undesirable material is taken in, feeding will soon be resumed. If the stimulus from the introduced material is rather strong the animal will cease feeding and will forcibly eject what water is in the branchial basket and atrium, and will remain closed for a considerable length of time, depending upon the strength of the stimulus.

In tunicates there is a ring of tentacles which interlace across the oral funnel which prevents the entrance of large particles. Such large particles as do find their way into the branchial basket are not incorporated in the mucus, but are in some way dropped from it into the branchial basket, and at intervals are forcibly ejected from the oral funnel by a sudden contraction of the body wall of the tunicate.

It is characteristic of animals which use mucus to entrap their food that they are able to drop from such mucus at least a portion of the undesirable material which is entrapped. Just how this is accomplished is not at present clear. It may be that the cilia which move the mucus can, by pressing outward through the mucus cause such particles to drop out. Many animals have specialized regions where the cilia perform this function. In the tunicates it is the cilia bordering the dorsal groove, in the pelecypods (future paper) it is the cilia of the lower edge of the gills and those of the labial palps, and in the echiuroid *Urechis* it is the outer cilia bordering the proboscis. In such regions the cilia are usually considerably larger than elsewhere.

When large particles strike the tentacles of the oral funnel they are usually blown away by a quick contraction of the mantle wall with little cessation of the feeding current. As most single tunicates hang vertically with the osteum downward one ejection movement serves to remove the large object. But, because of the separate action of individuals of a colony, a particle upon the surface of a colonial form which is fairly level may be bounced over the surface for some time before it is carried away by currents or is rolled over the edge. Since there is a constant current out of the atriopore (which in colonial forms may be common to several individuals), no particles find their way into the atrium and no tentacles are necessary, for if the current is stopped the atriopore closes.

As has been stated above, the cilia beat almost continually, and normally when the tunicate is not feeding most of the solid particles pass through the stigmata and out the atriopore. However, even when the branchial basket is not lined with the mucous sheets, some of the solid particles may find lodgment upon the ciliary tracts lining the branchial basket (particularly those of the endostyle, peripharyngeal grooves and dorsal groove), and will follow more or less the definite tracts. This is especially true of specimens that are handled or cut open, since they may secrete mucus along these grooves, whereas normally they would not do so. The mucus which carries such particles as are transported along these ciliary tracts may enter the esophagus or may be dropped into the branchial basket and be ejected through the oral funnel. The more or less abnormal performance just described has led to the erroneous ideas found in textbooks about the feeding of tunicates.

Ciliary Action

The ciliary action of *Ascidia californica* was studied in detail. The oral aperture, the atriopore, the cilia of the basket, and the cilia of the stigmata may all function independently of each other or they may all function together. The cilia of the stigmata may be stopped without stopping those of the basket. When the cilia of the stigmata cease vibrating they lie down against the edges of the openings, leaving the stigmata wide open. However, when the animal is contracted the edges of the stigmata are approximated and the openings closed. At such times, of course, the cilia are still and lie flat against the sides of the openings.

After the cilia have been stopped they resume their beating by starting to vibrate in a small circle at the center of the stigmatal opening, and this ring spreads towards either end of the opening until all are again beating. The beating cilia surrounding a stigmata remind one of an elongated wheel organ of a rotifer or a veliger larva. In the ascidian investigated the apparent movement was in an anti-clockwise direction as viewed from the outside.

There is no doubt that the cilia of the branchial bars, ridges and grooves actually hold and move mucus. The cilia seem partially to enter the sheet of mucus and force it forward. During part of the beat the cilia are more or less hooked into the mucus and this serves to hold it so that the cilia following are able to penetrate and in turn do their share of pushing and holding. This action of the cilia is further evidenced by the fact that the mucous sheet which is present on the inside of the basket when the animal is feeding has in it waves which correspond to the wave motion of the cilia. These waves in the mucus appear when water heavily laden with food is introduced into the oral funnel. As the food material collects in the mucous sheet it sometimes appears in streaks which are more accentuated as the mucus nears the dorsal groove.

SUMMARY

1. The feeding method of *Ciona intestinalis* and *Ascidia californica* (simple ascidians), and of *Diplosoma pizoni* (a colonial form) was investigated.

2. Tunicates feed by straining the solid material from a current of water as it passes through a thin film of mucus lining the branchial basket.

3. The mucus is constantly secreted at the endostyle and is continually moved to the dorsal groove in two sheets which line the interior of the basket. The dorsal groove forms the edges of the food-laden sheets into a thread which is passed posteriorly to the esophagus and enters it in an unbroken string. The peripharyngeal grooves serve to hold the anterior ends of the mucous sheets and move them around to the dorsal groove.

4. When a tunicate is not feeding, the inside of the branchial basket is not lined with mucus, and the solid materials pass out with the atrial current.

5. Some sorting is carried out by the cilia of the dorsal ridges. The cilia which line the openings of the stigmata, and whose vibration creates the current of water passing through the basket, may be stopped without stopping the cilia lining the basket or without closure of the oral aperture and atriopore.

6. After the cilia lining the stigmata have been stopped they commence to beat in what appears to be a ring at the center of the opening. The cilia of one side of a stigmata are in perfect synchronism with those of the opposite side of the opening, and, by the continual inclusion of other cilia, all finally vibrate and resemble somewhat an elongated wheel organ of a rotifer.

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TEST SECRETION IN TWO SPECIES OF FOLLICULINA

VIRGINIA C. DEWEY

(From the Arnold Biological Laboratory, Brown University and The Marine Biological Laboratory, Woods Hole, Massachusetts)

Introduction

The fact that *Folliculina* passes through a free-swimming stage at some point in its life cycle was recognized by Wright (1859) and by Claparède and Lachmann (1858–61). The former author observed the transformation of the free-swimmer of *F. producta* into the adult form, but did not discover the mode of origin of the "larva" nor any details of the metamorphosis. For this reason these observations were later questioned by Stein (1867), but they are completely confirmed by more recent work. That the larvae are formed as the result of cell division was pointed out by Möbius (1887), but the presence of a larva and an adult lying side by side in a single test he interpreted as a case of longitudinal division. In his figures he even shows an "umbilical cord" projecting laterally from the larva. *Folliculinas* do not, however, depart from the general rule of transverse division in ciliates as was pointed out by Sahrhage (1917). Andrews (1920) has shown that free-swimming larvae may also result from the dedifferentiation of an adult form.

Test secretion and metamorphosis are described by Penard (1919) for *F. boltoni,* a freshwater species. Too few details are given to decide whether or not this species passes through the same stages as are described for *F. producta* by Andrews (1923) or for *F. simplex* (previously called *F. ampulla*) by Fauré-Fremiet (1932). Observations on *F. aculeata* and *F. elegans* indicate that the process of test formation is somewhat different in these species. During the summer of 1936 at Woods Hole, while examining fresh preparations for specimens of *Folliculina,* a form was discovered of which no description could be found. The posterior part of the body resembled that of a *Folliculina* seen on the slide, but the anterior portion was drawn out into one long, slender, flexible process bearing membranelles only at the end. Circumstantial evidence pointed to the conclusion that these forms represented a stage in the life history of *Folliculina.* Definite proof of this theory was lacking, since the larvae did not remain in good condition long enough for complete transformation to take place. This stage has evidently been noted by Fauré-Fremiet (1936), but his descriptions are not detailed. In order to obtain more complete evidence concerning the significance of this stage in the life cycle, observations were resumed in 1939. A complete life history can now be given placing this stage in its proper sequence.

The classification of the members of the family Folliculinidae is still in a somewhat unsatisfactory state in spite of the fact that a number of investigators have given the matter a great deal of attention. The separation of the genus *Folliculina* into a large number of species on the basis of characteristics which are subject to considerable variation seems to be the rule. For this reason the classification given by Fauré-Fremiet (1936), in which several species which have a number of common characteristics are combined into a single species, is to be preferred. Two species, as described by this author, *F. aculeata* and *F. elegans,* seem to prevail at Woods Hole. The two are alike in many ways; the chief differences are in size, the presence or absence of pointed tips on the peristomeal lobes and in the pigmentation of the animal and of the test. Since all of these traits are subject to variation, so that one species may resemble the other very closely, it was difficult to determine which species was under observation. Certain organisms, however, presented all the criteria of one species or the other. Since the greater number of individuals observed were of a paler color and possessed the pointed peristomeal lobes characteristic of *F. aculeata,* this species is figured. The only point in which they differed from the descriptions of Fauré-Fremiet was in the color of the test which was often colorless or faintly blue. Both species have an ovoid nucleus, but, as in *F. boltoni,* it is often notched or bi-lobed.

METHODS

The organisms were collected by placing glass slides in crystallizing dishes containing quantities of the hydroid *Tubularia* and leaving them for several days in running sea water. The slides were then removed and placed in Petri dishes containing sea water. Observations were made with a binocular dissecting microscope, with the 16 mm. objective of a compound microscope or with a water immersion lens (40 ×). In order to maintain the organisms in good condition over periods of several hours, a stream of fresh sea water was run into the dish while on the stage of the microscope. The overflow was carried off by means of an inverted siphon. Temperature readings were regularly recorded and during the time that the observations were being made the temperature of the sea water varied from 21° C. to 24° C.

Observations

The complete life history of a larva resulting from cell division, from the time of departure from the test containing the sister cell until the adult form was attained, was followed for a number of individuals. In some cases free-swimming larvae were found and their subsequent history observed. In a few cases it was found that adult forms dedifferentiated into the larval form, left the test and settled down to secrete a new test.

After cell division (Fig. 1) the anterior individual, which will eventually leave the test as a free-swimming larva, remains for from 30 to 60 minutes in the test contracting and extending beside its sister cell, which remains attached and which has already begun the metamorphosis into the adult form. At each extension of the larva the anterior end is projected farther and farther out of the neck of the test until finally the whole organism is free. The swimming stage (Fig. 2) may last for from 15 to 90 minutes, during which time the larva swims slowly along the substratum or more rapidly near the surface of the water in the dish. At intervals it pauses to contract and extend itself at one spot and then swims on. Just before the larva settles down to secrete its test it may be seen to repeat this process of contraction and elongation a number of times in a single spot, changing direction each time it contracts until it has described a complete circle at least once and sometimes several times. It then flattens itself out on the substratum and its outlines become very irregular (Fig. 3). Occasionally larvae were seen secreting tests attached to the surface film of the water as described by Wright in 1859. This flattened stage lasts for several minutes, after which time it is difficult to dislodge the organism from the slide even with a fairly strong stream of water. It is at this time that a broad layer of cement substance is being secreted which will serve to attach the test to the substratum. During this stage and for the entire time that the test is being secreted the anterior end of the animal is raised above the rest of the body.

Having attached itself firmly, the animal now assumes a more regular ovoid form, becoming thicker and rounder (Fig. 4). Soon granules may be seen around the body among the cilia. These collect all around the periphery and harden to form the bottle-shaped part of the test.

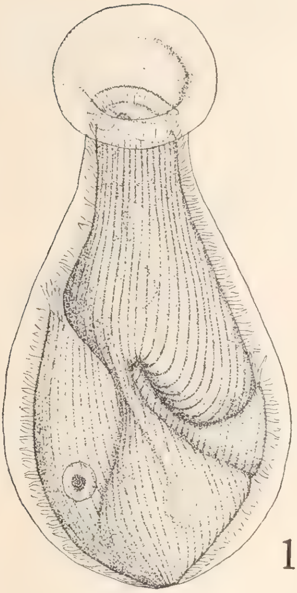
Figs. 1–4. *Folliculina aculeata.* × 300.

Fig. 1. Late division stage. Macronucleus not yet completely divided.

Fig. 2. Free-swimming larva.

Fig. 3. Larva flattened out on the substratum during the process of cement secretion.

Fig. 4. Beginning of the secretion of the test.



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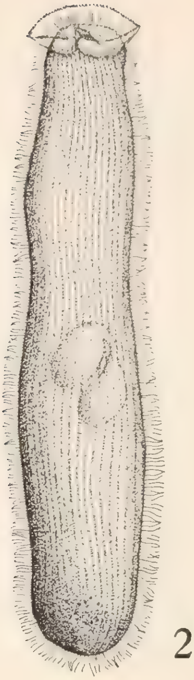

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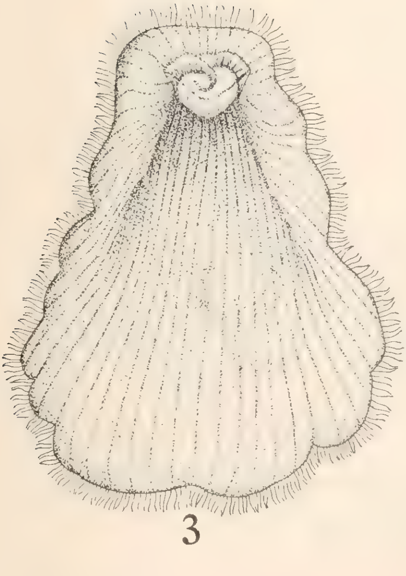

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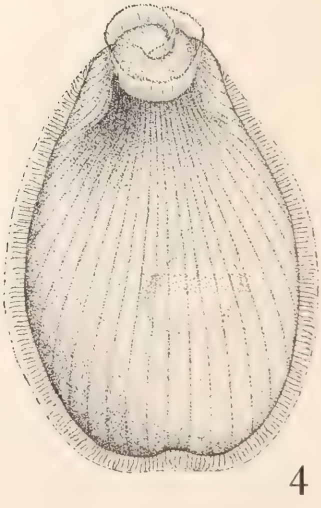

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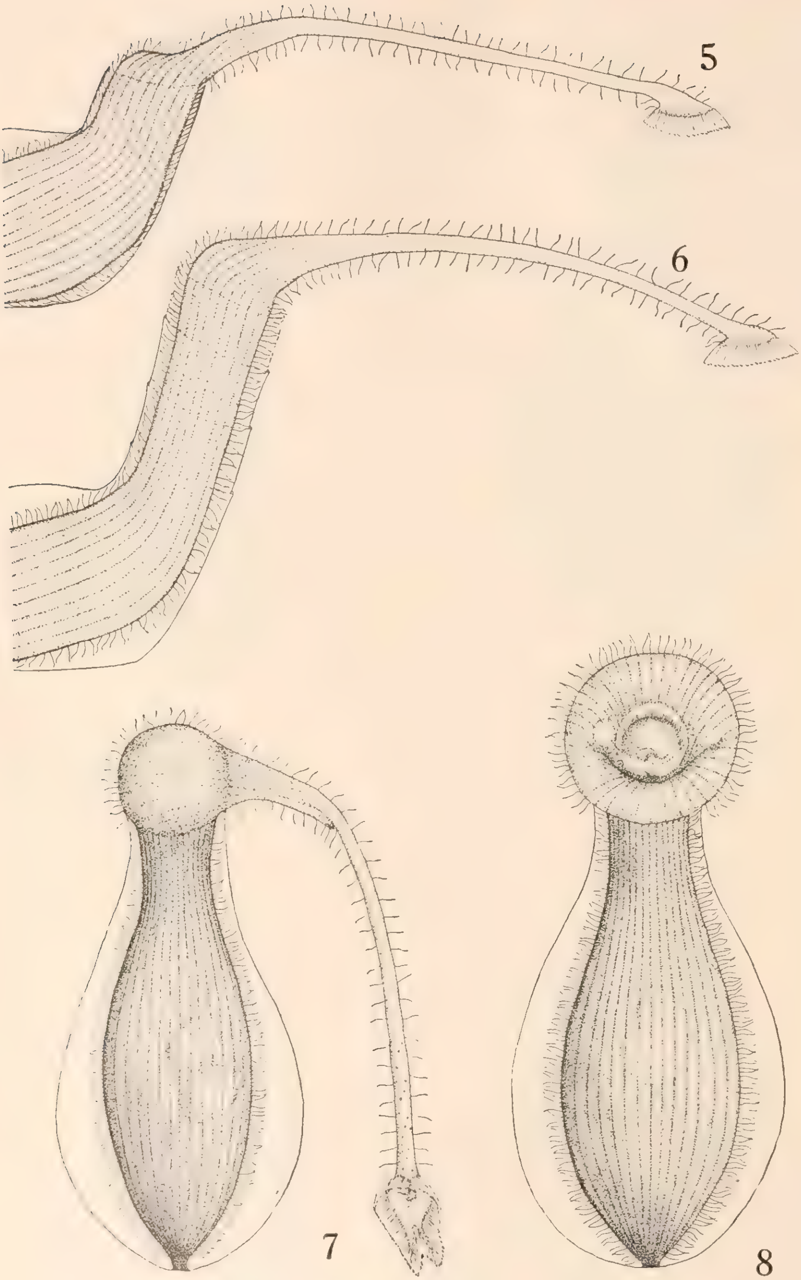

Figs. 5–8. *Folliculina aculeata.* × 300.

Fig. 5. Side view of larva with long proboscis at the beginning of secretion of the neck of the test.

Fig. 6. Same as Fig. 5. Neck secretion nearly completed.

Fig. 7. Same as Fig. 5. Top view.

Fig. 8. Beginning of secretion of the collar of the test.

The upraised anterior portion of the body secretes the base of the neck of the test. As the formation of the body of the test is completed, the anterior end of the animal elongates to form a proboscis-like projection of about the same length as the body. Upon the end of this proboscis the membranelles are borne (Figs. 5, 7). The proboscis is seen to sweep about in circles and while it is present the neck of the test becomes

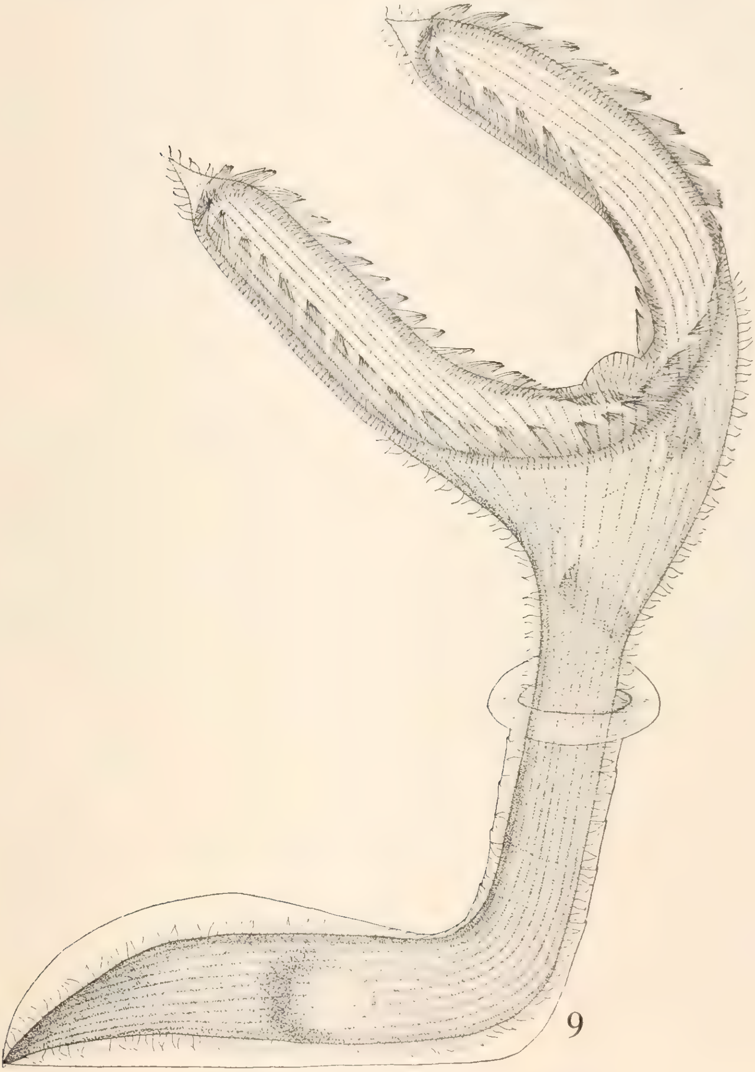

FIG. 9. Adult *Folliculina aculeata.* × 300.

longer and the spiral rings are laid down (Fig. 6). This stage is observed about 50 to 75 minutes after the larva has become attached and lasts about 70 to 100 minutes.

It is difficult to state whether or not this stage has previously been described. Penard (1919) states that Lachmann observed the fixation of a larva of *F. elegans* " after which . . . at the anterior end a membranous extension appeared, which I should be disposed to consider as moribund phenomena " (p. 312). Upon referring to the original paper this stage is described as presenting an " épanouissement membraneux " (p. 219). This seems clearly to refer to the next stage to be described in this cycle. It is improbable that this is an abnormality in the development, since this stage was observed in every larva whose development was followed. In the metamorphosis of *F. producta* and *F. simplex* this stage is apparently lacking (Andrews, 1923; Fauré-Fremiet, 1932). The figures of Fauré-Fremiet (1936) cannot be definitely identified with this stage.

Upon completion of the tubular part of the neck of the test, the proboscis is retracted and a double fold of cytoplasm is extended around the opening to form a collar (Fig. 8). During the 55 to 115 minutes that this stage lasts the collar of the test is secreted. When this has been finished the cytoplasm frees itself from the rim of the collar and the ragged edges are withdrawn into the test. In this contracted state the animal remains for from 3 to 5 hours. The changes occurring during this time are described in detail by both Andrews and Fauré-Fremiet and seem to be essentially similar in all forms. At the end of this period the animal protrudes from the test the long peristomeal lobes characteristic of the adult form (Fig. 9) The entire process of test secretion and morphogenesis may take from 4½ to 8¾ hours.

A single small larva of a yellow color, probably *F. viridis,* was discovered on the slide in the early stages of test secretion and was followed through to the adult stage. The development followed that of *F. aculeata* and *F. elegans,* although the proboscis was relatively shorter than in these species and the peristomeal lobes of the adult were smaller and more rounded.

I acknowledge gratefully the advice and encouragement of Dr. George W. Kidder.

SUMMARY

1. Test secretion and metamorphosis in *Folliculina aculeata* and *Folliculina elegans* are described.

2. These processes may be divided into the following six stages:

a. The free-swimming stage.

b. The stage of cement secretion.

c. The secretion of the body of the test.

d. The secretion of the neck of the test, during which process the animal puts forth a long, proboscis-like projection. This stage is, as far as is known, peculiar to *F. aculeata*, *F. elegans* and *F. viridis* and has not been described before in detail.

e. The secretion of the collar of the test.

f. Formation of the peristomeal lobes characteristic of the adult.

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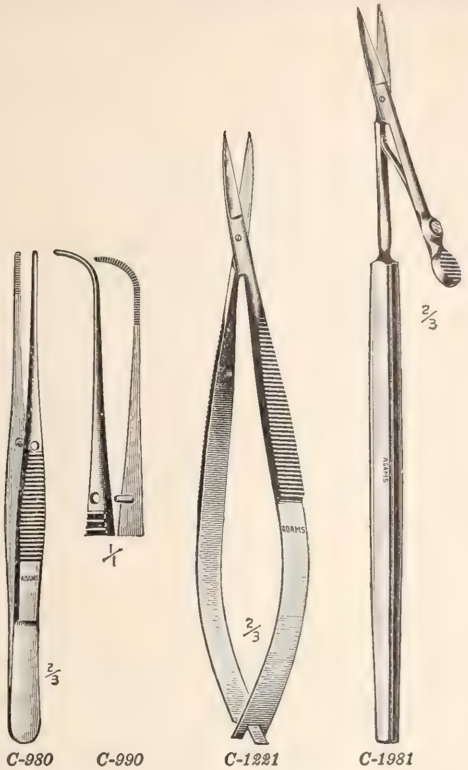

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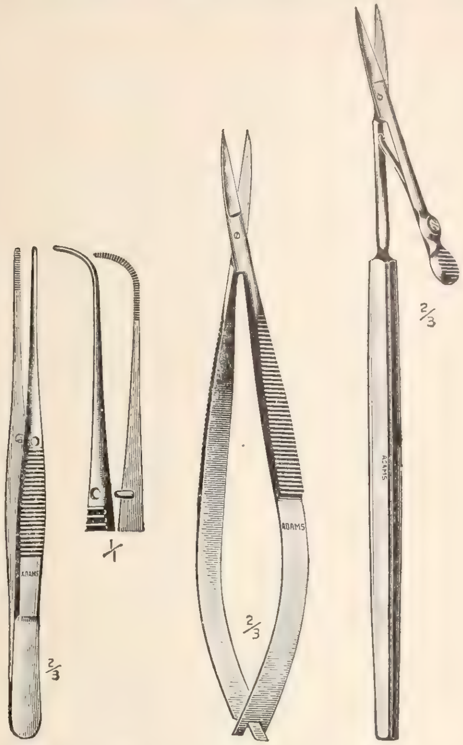

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